REMARKS

On page 2 of the Office Action, the Examiner maintains the rejection of Claims 9-10 under 35 U.S.C. § 103 as being unpatentable over Slavin et al in view of Ildstat et al, Zhang et al and Sachs.

The Examiner acknowledges that TBI taught in Slavin et al was conducted at a dose of 4.0 Gy, which is much less than claimed in the present application. However, it is the Examiner's position that one skilled in the art would appreciate that a higher dose of TBI might be beneficial as taught by Sachs (*sic* Ildstad et al). Thus, the Examiner contends that the present invention would have easily been achieved by combining Slavin et al, which discloses TLI and TBI, and also discloses performing TLI and administration of bone marrow cells (BMC) within one day, with Ildstad et al which teaches TBI of higher values.

For the following reasons, Applicants respectfully traverse the Examiner's rejection.

Slavin et al teaches that TBI is less preferred to TLI (see column 8, lines 63-65). Further, with reference to Figure 2 thereof, it is apparent that merely adjusting the dose of irradiation does not improve both survival % and SKIN ACCEPTANCE % (see Figure 2 of Slavin et al; and EXAMPLE 9 from column 32, line 11 to column 34, line 50 thereof). Specifically, Figure 2 of Slavin et al shows that survival % activity actually decreases as the dose of irradiation increases.

Thus, in view of Figure 2 of Slavin et al, wherein increasing the dose of irradiation reduces survival %, a person skilled in the art would never think of increasing the dose of TBI in the method of Slavin et al.

Hence, one skilled in the art would not have been motivated, based on the teachings in Slavin et al to combine Slavin et al with Ildstad et al to use a dose of 6.5 Gy, as claimed in the present application. That is, one skilled in the art would have considered it undesirable to combine TBI with irradiation at values higher than 4.0 Gy, as based on Slavin et al, one skilled in the art would have considered such high values would have reduced survival %.

The Examiner notes Applicants argument that Ildstad et al relates to a technique using mixed chimerism, not fully allogenic chimerism. However, it is the Examiner's position that Claims 9-10 do not recite a technique using fully allogenic chimerism.

The Examiner is requested to note that Claim 9 recites TBI of at least 6.5 Gy. As is clear from Figure 1 of Ildstad et al, recipient cells are completely removed using a TBI of 6.5 Gy. Moreover, the present invention involves administering of only donor cells to a recipient, and not recipient cells. Thus, Claims 9-10 are clearly directed to fully allogeneic chimerism, not mixed chimerism as taught in Ildstad et al.

In addition, none of Slavin et al, Zhang et al and Sachs et al teaches or suggests the importance of fully allogenic chimerism for achieving engraftment. Thus, the present invention could not have easily been conceived from any of Slavin et al, Ildstad et al, Zhang et al and Sachs et al, or a combination thereof.

The Examiner also notes Applicants' argument that Figure 7 of Ildstad et al does not show 100% acceptance of skin grafts after 30 days. However, the Examiner contends that Ildstad et al teaches that grafts were followed for a

minimum of 35 days and there is no data that shows that after that time the grafts were rejected. Further, the Examiner draws Applicants' attention to column 17, lines 5-25 of Ildstad et al where it is disclosed that the allogenic engraftment was reliably achieved in 100%.

The Examiner is requested to note that the cited teaching in Ildstad et al merely indicates that allochimerism can occur at around 6 Gy or more.

Ildstad et al argues that in the case of donor skin grafting, engraftment and tolerance were achieved (see from column 21, line 58 to column 22, line 8; and Figure 7). However, in Figure 7 thereof, it is recipient skin grafting which still maintains 100% survival after 35 days. The percent survival for donor skin grafting was decreased to about 90% after 19 days. In other words, the donor grafts were clearly rejected.

Ildstad et al teaches the advantages of using TBI in engraftment (column 9, lines 15-18). Ildstad et al states that stable engraftment of allogeneic donor bone marrow cells was achieved (column 9, lines 6-11), and the degree of donor chimerism achieved was >90% (column 19, lines 15-46). However, with only about 90% of donor cells when administering bone marrow cells, i.e., in the case of mixed chimerism, the donor cells are gradually decreased, and ultimately the grafts are rejected (see Figure 1 of Hayashi et al, *Stem Cells*, 18:273-280 (2000)); and page 517, from line 19 in the left column to line 3 in the right column of Ikebukuro et al, *Transplantation*, 73(4):512-518 (2002)). In order to prevent rejection of donor cells in mixed chimerism, it is necessary to administer donor lymphocyte (T cells) to a receipt to convert mixed chimerism to full donor chimerism (see the

Abstract of Kim et al, *Blood*, 103(2):732-739 (2004)). For this reason, in the example of mixed chimerism of Ildstad et al, wherein "the degree of donor chimerism achieved was >90% (column 19, lines 15-45)", the grafts will ultimately be rejected.

In contrast, the present invention succeeded in engraftment of skin for over 13 weeks (see page 28, lines 10-16 of the present specification). Further, Applicants discovered the importance of fully allogenic chimerism, rather than mixed chimerism, for achieving engraftment. For this reason, Applicants constructed a method of inducing immunological tolerance whereby engraftment can be achieved for a longer period of time, the method comprising subjecting a recipient to TBI for completely suppressing recipient cells, and administerining donor cells which do not mix with the recipient cells of the recipient.

Thus, the method of the present invention is unpredictable from Ildstad et al, wherein mixed chimerism is induced, and the grafts will ultimately be rejected.

As to Zhang et al, this reference teaches that through portal vein administration, more donor cells tend to be trapped in the liver than through intravenous administration (see the Abstract). However, Figure 10 thereof shows that the allogeneic BMC in the liver peaks on the 10^{th} day, but therafter declines sharply. Hence, it is likely that the intraportally administered donor cells decrease before engraftment is achieved, and subsequently the cells are rejected, as with the case of intravenous administration in Zhang et al. Thus, prior to the present invention, it was unpredictable whether portal vein administration would actually bring about successful skin engraftment.

As to Sachs et al, this reference may teach the administration of TBI and BMC within one day. However, Sachs et al teaches that TBI may only be performed with a low dose, of between 1.0 and 4.0 Gy (see column 3, lines 49-58 thereof). As it is clear from Figure 1 of Ildstad et al, allogeneic chimerism cannot be achieved at TBI of 4.0 Gy. Because allogeneic chimerism is not achieved in Sachs et al, it is highly likely that the grafts were ultimately rejected, based on the aforementioned reason given in Hayashi et al (*Stem Cells*, 18:273-280 (2000)), Ikebukuro et al, (*Transplantation*, 73(4):512-518 (2002), and Kim et al (*Blood*, 103(2):732-739 (2004)), that the donor cells gradually decrease, and the grafts will ultimately be rejected.

Moreover, Zhang et al does not disclose irradiation. Therefore, even by combining the portal vein administration of Zhang et al with irradiation disclosed in Slavin et al, Ildstad et al, and Sachs et al, one skilled in the art would not have predicted (1) how the period during which the donor cells remain in the liver is affected, (2) whether the donor cells are trapped in the liver, and (3) whether the effect of inducing immunological tolerance through portal vein administration is improved.

Thus, the advantageous method of inducing immunological tolerance (as claimed in the present invention) could not have easily been predicted by combining Zhang et al with Slavin et al, Ildstad et al and Sachs et al.

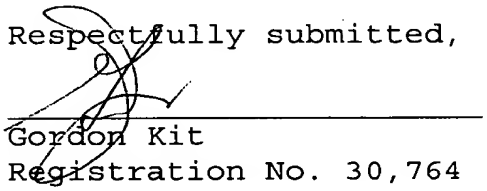
Accordingly, Applicants respectfully submit that the present invention is not taught or suggested by Slavin et al alone or when combined with the teachings of Ildstad et al and Zhang et al, and Sachs and in any event, such a combination can only be made in hindsight, which is legally improper.

Thus, Applicants request withdrawal of the Examiner's rejection.

In view of the arguments set forth above, reexamination, reconsideration and allowance are respectfully requested.

The Examiner is invited to contact the undersigned at the telephone number listed below on any questions that might arise.

Respectfully submitted,



Gordon Kit
Registration No. 30,764

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON OFFICE
23373
CUSTOMER NUMBER

Date: February 28, 2006

reference 1

Long-Term (>1 year) Analyses of Chimerism and Tolerance in Mixed Allogeneic Chimeric Mice Using Normal Mouse Combinations

HARUKI HAYASHI,[a,b] JUNKO TOKI,[a] LIAN ZHEXIONG,[a] KIKUYA SUGIURA,[a]
KYOICHI INOUE,[b] SUSUMU IKEHARA[a]

[a]First Department of Pathology, [b]Third Department of Internal Medicine,
Kansai Medical University, Osaka, Japan

Key Words. *Mixed allogeneic chimeras · Pancreas allografts · Mice · Tolerance*

ABSTRACT

We examined the induction of tolerance using pancreas allografts over the long term (>1 year) in mice for the human application of mixed allogeneic bone marrow transplantation (BMT). T cell-depleted BM cells (BMCs) of C57BL/6 (B6) and C3H/He (C3H) mice were transplanted at various ratios into lethally irradiated B6 mice. The percentages of C3H cells in the chimeric mice gradually decreased, finally declining to only a small percentage, except when the ratio of donor to recipient BMCs was 100:1. However, despite the marked decreases in C3H-type cells, all the pancreas allografts of C3H mice were accepted when more than 1% C3H cells were detected in the peripheral blood. To examine the relationships between percentages of transplanted donor cells and acceptance of pancreas allografts, various percentages of donor and recipient BMCs (5% to 30%) were transplanted. It was found that more than 10% donor cells were necessary for the pancreas allografts to be accepted. In vitro assays for mixed lymphocyte reaction and generation of cytotoxic T-lymphocytes revealed that spleen cells in chimeric mice accepting pancreas allografts are tolerant to both host-type and donor-type major histocompatibility complex (MHC) determinants, but show a vigorous responsiveness to third-party MHC determinants. Since donor-type hemopoietic stem cells (HSCs) were detected in the BM and the liver of the chimeric mice, donor-derived HSCs and donor-derived hematolymphoid cells are responsible for the induction of tolerance. It should be noted that the percentage of donor-type HSCs is higher in the liver (6.2%) than in the BM (0.9%). *Stem Cells 2000;18:273-280*

INTRODUCTION

In humans, organ allografts require the use of immunosuppressants to prevent graft rejection. In mice, we have previously shown that allografts of organs such as the liver and pancreas, in conjunction with allogeneic bone marrow transplantation (BMT) from the same major histocompatibily complex (MHC) donors, can induce permanent tolerance without using immunosuppressants [1, 2]. *Ildstad* and *Sachs* have established a system of mixed allogeneic chimerism by carrying out mixed allogeneic BMT and have demonstrated that mixed allogeneic BMT can be used for organ transplantation [3]. Mixed allogeneic chimerism has several advantages over fully allogeneic chimerism. The presence of syngeneic (or autologous) bone marrow cells (BMCs) appears to provide the necessary cells to overcome the impaired immunologic functions and prevent the graft-versus-host-disease (GVHD) observed in fully allogeneic chimeras, while the allogeneic BM elements appear to be responsible for the induction of donor-specific tolerance. *Starzl et al.* have found in humans that there are some cases in which liver allografts survive without using immunosuppressants, and that, in such cases, a small number of hemopoietic cells derived from the transplanted organs are detected: they described this as microchimerism [4]. In mice, mixed allogeneic chimerism can be achieved by carrying out mixed allogeneic BMT. In this condition, donor-specific tolerance can be induced. However, for human application to organ transplantation, long-term observation using allografts of organs other than the skin is necessary, since we have very recently found that the skin is not rejected after donor-derived Langerhans' cells have been replaced by

Correspondence: Susumu Ikehara, M.D., Ph.D., First Department of Pathology, Kansai Medical University 10-15 Fumizono-cho, Moriguchi City, Osaka 570-8506, Japan. Telephone: 81-6-6993-9429; Fax: 81-6-6994-8283; e-mail: ikehara@takii.kmu.ac.jp
Received May 12, 2000; accepted for publication May 23, 2000. *1066-5099/2000/$5.00/0*

host-derived Langerhans' cells, even when host-derived hematolymphoid cells become dominant (manuscript in preparation). In addition, we have found that the pancreas is more immunogeneic than the skin (manuscript in preparation), although it has been thought that the skin is the most immunogeneic tissue. Therefore, we examine the induction of tolerance, chimerism, and pancreas allograft acceptance in normal mouse combinations over a long term (>480 days after transplantation). In the present study, we show that mixed allogeneic BMT can be used for organ allografts, although the number of allogeneic hematolymphoid cells gradually decreases.

Materials and Methods

Mice

Female C57BL/6J (B6: H-2^b), C3H/HeN (C3H: H-2^k), and BALB/c (H-2^d) mice (five to eight weeks old) were purchased from SLC (Shizuoka, Japan), and raised at the Kansai Medical Animal Care Center.

Mixed Allogeneic BMT ([B6 + C3H]→B6)

Mixed allogeneic chimeric mice were prepared as previously described [3]. Briefly, inbred B6 female recipient mice were lethally irradiated with 10 Gy from a ^{137}Cs source (Gammacell 40 Exactor, Nordion International Inc.; Kanta, Ontario; http://www.MDSNordion.com). The BM was flushed from the femurs of the B6 and C3H donor mice with RPMI 1640 using a 23-gauge needle. The BMCs were gently resuspended with a 21-gauge needle, and the suspension was filtered through a nylon mesh. The BMCs were then washed at 1,500 rpm for 5 min, resuspended in RPMI, and counted. T cells in the BMCs were depleted using anti-Thy1.2 monoclonal antibody (mAb) (F7D5, Olac; Bicester, England) at 4°C for 30 min. They were then washed and resuspended in guinea pig complement at a 1/16 dilution in RPMI (1×10^7 cells/ml in diluted guinea pig complement) at 37°C for 40 min. The BMCs were then washed twice and resuspended in RPMI at an appropriate concentration for injection of 0.5 ml of final volume per mouse. The recipient mice were reconstituted within 12 to 24 h after irradiation with 1×10^7 BMCs mixed at various ratios (B6: C3H = 2:1, 1:1, 1:3, 1:10, or 1:100, and B6 or C3H only). In some experiments, the recipient mice were reconstituted in the ratios of 5% (18:1), 10% (9:1), 20% (4:1), and 30% (7:3), respectively.

All recipients were evaluated for the presence of clinical GVHD as manifested by weight loss, alopecia, ruffled fur, diarrhea and a decreased level of activity associated with a "hunched over" appearance. In addition, histological evidence for GVHD was evaluated using samples of the skin, liver, intestine and hematolymphoid organs (the spleen, lymph nodes, thymus and BM).

Transplantation of Fetal Pancreas Tissue

The procedure was as described previously [5]. Briefly, recipients were anesthetized with an i.p. injection of somnopentyl (0.1mg/g body weight). Light ether anesthesia was used, if necessary, during the operation. A vertical incision was made in the lumbar region, and the underlying kidney gently pulled out of the abdomen.

A longitudinal incision was made in the renal capsule. The edge of the incised capsule was lifted up with fine forceps, and the fetal pancreas grafts placed under the capsule, and pushed away from the incision. The fetal pancreas was used because it contains more islets, but less exocrine glands, than the adult pancreas. The kidney was replaced within the peritoneal cavity and abdominal muscles, and the skin incision closed with silk sutures. It required about 10 min for a single engraftment.

Cell Preparation

Peripheral blood (PB) was collected into heparinized plastic vials from the orbital cavity. After mixing, the suspension was layered over 1.5 ml of room temperature lymphocyte separation medium (Lympholyte-M; Cedarlane; Hornby, Ontario; http://www.cedarlanelabs.com) and centrifuged at 3,000 rpm for 30 min at 23°C. The lymphocyte layer was aspirated from the saline-Lympholyte-M interface and washed with medium.

BMCs were collected from the femurs of recipient mice, as previously described [5]. The spleen and lymph nodes were gently teased on a fine steel mesh, and cell suspensions washed twice in RPMI-1640 medium (Nissui; Tokyo, Japan; http://www.nissui.co.jp/top.html), and finally suspended in medium containing 10% fetal calf serum (FCS) (HyClone Laboratories; Logan, UT; http://www.hyclone.com).

Hepatic mononuclear cells (MNCs) were obtained as follows: the liver was perfused in situ via the portal vein with 10 ml of Dulbecco's phosphate-buffered saline (PBS) and 10 ml of prewarmed (38°C) PBS(-) containing 150 U/ml Type IV collagenase (Sigma Chemical Co.; St. Louis, MO; http://www.sigma-aldrich.com). The liver was removed and cut into small pieces. The tissue was transferred into a 50-ml tube, dispersed by pipetting, and added to 40 ml of PBS containing 2% FCS. The cell suspension was centrifuged at 35× *g* for 1 min at 4°C to remove tissue debris and parenchymal cells. The hepatic MNCs in the supernatant were washed three times at 250× *g* for 5 min. The hepatic MNCs in the pellet of the last centrifugation were suspended in 2 ml of 31.5% Percoll solution (Pharmacia; Uppsala, Sweden; http://www.pnu.com), layered onto 2 ml of 70% Percoll solution in a 15-ml tube, and covered with 2 ml of PBS. After the centrifugation at 450× *g* for 20 min, the hepatic MNCs in the lower interface were

removed and washed twice. The recovery for hepatic MNCs was about 31%, and the contamination of PB MNCs was less than 1%.

Flow Cytometry

Fluorescein isothiocyanate (FITC)-coupled anti-H-2K^k (030-39F) and H-2K^b (030-11F) mAbs from Meiji Institute of Health Service (Odawara, Japan) and phycoerythrin (PE)-coupled anti-H-2K^b mAb from PharMingen (San Diego, CA; http://www.pharmingen.com) were used for determining the percentage of cells bearing MHC class I (H-2K^b and H-2K^k) surface markers in the PB lymphocytes, BM, spleen, lymph nodes, and liver. PE-coupled anti-CD4, CD8, B220, Mac-1, Gr-1, and CD71 mAbs from PharMingen were used for characterizing the donor-derived BM and liver MNCs. mAbs against erythroid lineage cells (TER119) were kindly donated by *T. Kina* (Chest Disease Institute; Kyoto University, Kyoto, Japan; http://www.Kyoto-u.ac.jp). The cells were suspended in PBS containing 2% FCS plus sodium azide, then incubated on ice with the appropriate mAbs for 30 min and analyzed by flow cytometry on FACScan (Becton Dickinson & Co.; Mountain View, CA; http://www.bd.com) equipped with logarithmic scales.

Mixed Lymphocyte Reaction (MLR)

Triplicate cultures from four chimeric mice and four control mice were set up in round-bottom 96-well microwell trays (Corning Inc.; Corning, NY; http://www.corning.com). Each well contained 2×10^5 responder cells and 10^5 stimulator cells in a total of 0.2 ml of RPMI 1640 medium supplemented with 2mM L-glutamine, penicillin (100 units/ml), streptomycin (100 mg/ml) (Sigma-Aldrich; St. Louis, MO), 10% heat-inactivated FCS, and 50 mM 2-mercaptoethanol (2-ME; Wako; Osaka, Japan). Stimulator cells were irradiated with 20 Gy. The cultures were incubated for 96 h in a humidified 5% CO_2 atmosphere. [^{3}H] thymidine (0.5 mCi) was introduced during the last 4 h of the culture period. [^{3}H] incorporated into trichloroacetic acid-insoluble materials was measured using a liquid scintillation counter.

Generation of Cytotoxic T-Lymphocytes (CTLs)

Responder cells (7.5×10^6) and stimulater cells (2.5×10^6) were cocultured in RPMI 1640 medium containing 10% heat-inactivated FCS, supplemented with 50 mM 2-ME. Cultures were incubated for five days at 37°C in a 5% CO_2 atmosphere. Cells of the cell lines (P815 [H-2^d], EL-4 [H-2^b] and X5563 [H-2^k]) were used as target cells. These cells were labeled by incubation for 1 h at 37°C with 100 mCi of Na251CrO4 (NEN Life Science Products Inc.; Boston, MA; http://w.nenlifesci.com). After washing three times, labeled cells (5×10^4) were mixed with effector cells in 100 ml of RPMI 1640 medium in round-bottom microwells and incubated at 37°C in 5% CO_2 for 4 h. The Titertek supernatant system was used for determination of released radioactivity of ^{51}Cr.

Percent-specific lysis was calculated as ([experimental release-spontaneous release]/[maximal release-spontaneous release]) × 100. In the analyses, spleen cells were pooled from mice, and the analyses were performed in triplicate.

Histology

Recipient mice were sacrificed each month after engraftment. The grafts were easily identified as a rounded white swelling on the surface of the kidney. The acceptance and growth of the grafts was assessed using a dissecting microscope, and the kidneys from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for the histological examination.

The data shown in the figures and tables are representative data since reproducible results were obtained.

Results

Analyses of Chimerism

Chimerism was analyzed by flow cytometry using FITC-conjugated anti-class I (H-2^b and H-2^k) mAbs. As shown in Figure 1, mixed chimerism was observed in almost all chimeric mice except the mice reconstituted at a host:donor ratio of 1:100. Donor-type T cells, B cells and macrophages were detected (data not shown). Since there was no significant difference between the spleen, lymph nodes and PB, the degree of chimerism was shown in the PB. As shown in Figure 1, donor-type cells (H-2K^k) gradually decreased in all mice except for the mice reconstituted with a ratio of 1:100. However, it should be noted that donor-type cells did not completely disappear. Even at 15 months, 38.0%, 12.0%, 8.2% and 6.2% of donor-type cells were detected in mice reconstituted with the ratios of 1:10, 1:3, 1:1 and 2:1, respectively. Although observations were continued for 16 months, no symptoms of GVHD were clinically or histologically observed in the mixed allogeneic chimeric mice. Complete replacement with donor-type cells was observed in mice reconstituted at a ratio of 1:100.

Graft Acceptance

The pancreas tissues transplanted under the capsules of the kidneys in the recipient mice were histologically examined at various time points. As shown in Figure 2, the grafts were accepted by the recipient mice with established mixed chimerism, but rejected in mice without mixed chimerism. Even in mice with a markedly decreased percentage of donor-type cells, no destruction of grafted pancreatic tissue was observed when more than 1% of donor cells were detected in the PB. Immunohistochemical studies revealed

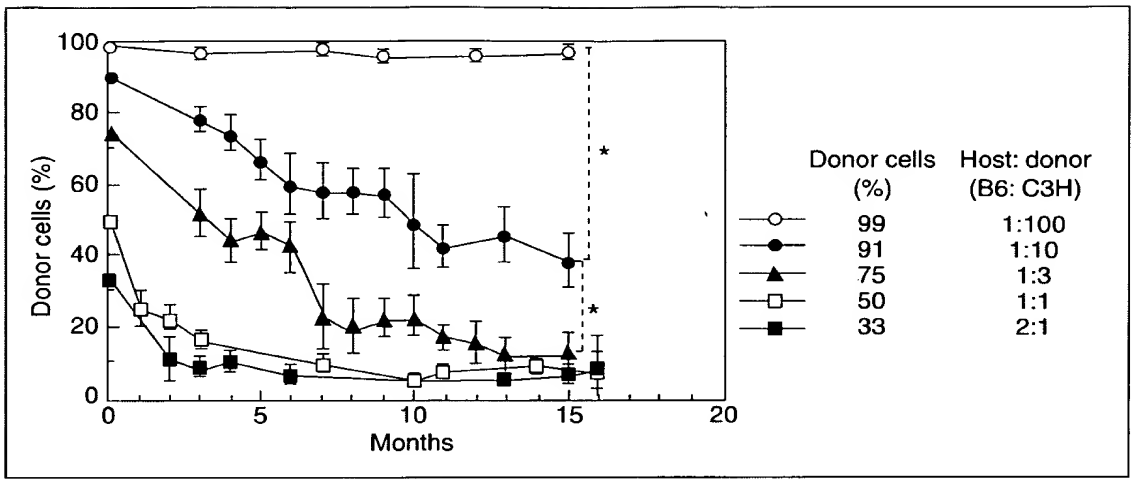


Figure 1. Percentages of donor (H-2K^k)-derived cells in the PB of mixed chimeras by two-color FACS analyses. *B6 hosts were lethally irradiated (10 Gy) and then reconstituted with a mixture (total 1 × 10^7) of T cell-depleted syngeneic and allogeneic BMCs with various proportions, as described in* ***Materials and Methods****. PB was collected from the mice in each group every month after treatment. The groups of 1:100, 1:10, 1:3, 1:1 and 2:1 consisted of 10, 15, 15, 13, and 7 mice, respectively. Statistical analyses were performed by Mann-Whitney U-test: p < 0.005, 1:10 versus 1:100 and 1:3.*

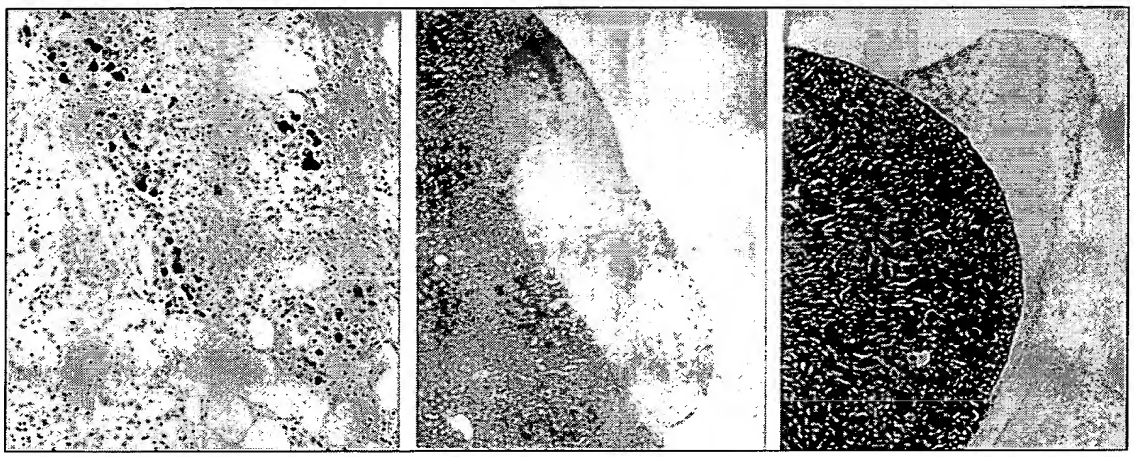


Figure 2. Histological findings of the grafts. *Recipients were transplantated with the fetal pancreas under the capsule of the kidney. The mice were sacrificed 3, 6, and 12 months after transplantation, and the grafts were stained with hematoxylin and eosin. In this figure, representative pictures in mice (one year after transplantation) reconstituted with a 1:3 ratio are shown; the mixed chimeric mouse (B6 + C3H[1:3]→C3H) accepts C3H pancreatic tissue (islets) with insulin-producing cells (brown) (left), whereas such a chimeric mouse rejects the third-party (BALB/c) pancreas tissue (middle). (B6→B6) mouse rejects C3H pancreas tissue (right). The rejected pancreatic tissues were replaced by fibrous tissue (middle) or adipose tissue (right) within one month after transplantation.*

the presence of insulin-producing cells (brown) in the engrafted islets (Fig. 2 left).

Induction of Tolerance

The next step was to examine whether donor-specific tolerance is induced in mixed allogeneic chimeric mice using MLR and CTL assays.

MLR was performed to examine the induction of tolerance. Representative data are shown in Figure 3. Twelve months after transplantation, 97.13%, 45.8% and 14.3% of donor cells were detected in mice reconstituted with the ratios of 1:100, 1:10, and 1:3, respectively. All these chimeric mice showed low responses to both donor- and host-type stimulators, although they showed significantly

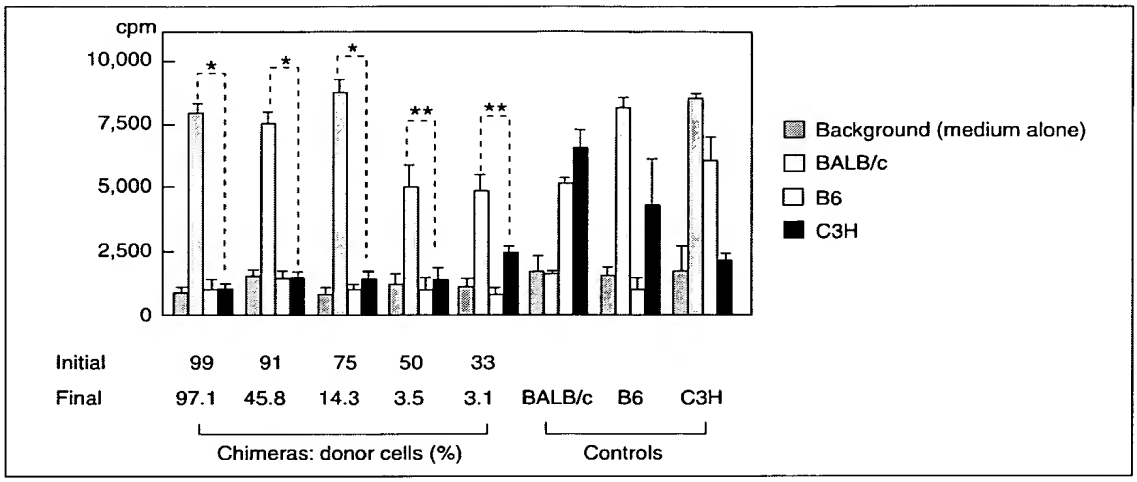


Figure 3. MLR of spleen cells in B6, C3H, BALB/c, and ([B6 + C3H]→B6) chimeric mice one year after treatment. *More than three experiments were carried out, and reproducible results were obtained. Representative data are therefore shown. Chimeric mice respond only to third-party (BALB/c) stimulator. Asterisks represent* p *values of responses to donor-type stimulators versus third party by* t*-test.* *p < *0.0001.* **p < *0.005.*

high responses to the third-party (BALB/c) cells (Fig. 3). Moreover, when 3.5% and 3.1% of donor cells were detected 12 months after transplantation in mice reconstituted at ratios of 1:1 and 2:1, these chimeric mice showed significantly high responses to the third-party (BALB/c) cells, whereas they showed low responses to both donor- and host-type stimulators (Fig. 3).

Table 1. Cytotoxic T-lymphocytes from spleen cells of chimeric mice are tolerant to both host-type and donor-type MHC determinants

Mouse	% specific lysis (E/T = 6)[a]		
	P815 ($H\text{-}2^d$)	EL4 ($H\text{-}2^b$)	BW5147 ($H\text{-}2^k$)
BALB/c ($H\text{-}2^d$)	0	43	79
C57BL/6 ($H\text{-}2^b$)	76	2	75
C3H/HeN ($H\text{-}2^k$)	81	48	2
(B6→B6)	75	2	76
(B6:C3H[1:100]→B6)	63	1	1*
(B6:C3H[1:10]→B6)	80	2	2*
(B6:C3H[1:3]→B6)	51	1	1*
(B6:C3H[1:1]→B6)	33	0	1*
(B6:C3H[2:1]→B6)	32	0	8*

[a]Spontaneous releases and maximum releases were 1,594 and 26,204 cpm in P815, 1,944 and 29,826 cpm in EL-4, and 1,345 and 18,515 cpm in BW 5147, respectively.

**p* value of cytotoxic activity to donor-type versus third party by *t*-test: $p < 0.0001$.

Similar results were obtained in CTL assays (Table 1). The spleen cells of the chimeric mice showed cytotoxic activity to third-party ($H\text{-}2^d$) cells, but not to host-type or donor-type cells.

Critical Doses of Donor Cells for Allograft Acceptance

To determine the critical doses of initially transplanted donor cells for allograft acceptance, we next carried out BMT using various ratios of donor and recipient BM cells; the ratios of donor BM were adjusted to 5%, 10%, 20%, or 30%. In addition, pancreatic tissue transplantation was performed at the same time. As shown in Table 2, the grafts were accepted by the recipient mice reconstituted with 10%, 20%, or 30% donor BM cells, but not 5% donor BM cells. Mixed chimerism was observed in recipient mice reconstituted with 10%, 20% or 30% donor BM. The induction of tolerance in these mice was examined by MLR. As shown in Figure 4, chimeric mice reconstituted with 5% donor BM cells, in which the grafts had been rejected, showed high responses to C3H mouse MHC determinants, while chimeric mice reconstituted with 10%, 20%, and 30% donor BM cells, in which the grafts were accepted, showed low responses to C3H mouse MHC determinants. From these results, it can be concluded that the presence of chimerism in the PB is useful for evaluating the induction of tolerance; it seems that more than 10% donor BM cells are initially necessary to prevent the rejection of donor pancreas tissue.

Table 2. Relationship between acceptance of donor pancreatic grafts and percentages of initially transplanted donor cells in chimeric mice[a]

Initial donor (C3H) cells (%)	After six mon		
	Mouse *n*	Graft	C3H (%)
5	1	–	0
	2	–	0
	3	–	0.1
10	4	+	1.5
	5	+	3.8
	6	+	2.6
20	7	+	5.8
	8	+	8.2
	9	+	23.9
30	10	+	11.0
	11	+	12.1

[a]Chimeric mice were perpared as described in **Materials and Methods**. Six months after BMT, the graft acceptance was examined, and the percentages of the donor cells in the peripheral blood were analyzed using a FACS.

Allogeneic Hemopoietic Stem Cells (HSCs) in the BM and Liver

Since donor-type T cells, B cells, and macrophages are observed in the BM and liver of the recipient mice for an extended period after transplantation, it is conceivable that donor-type HSCs are present in the recipient mice. We therefore analyzed whether donor-type HSCs are present in the BM and liver using a fluorescence-activated cell sorter (FACS), as previously described [6]. As shown in Figure 5, $Lin^-/CD71^-/H\text{-}2^{high}$ cells, which are pluripotent HSCs, as previously described [6-8], were observed in the BM and liver; it should be noted that the percentage of donor HSCs is higher in the liver (6.2%) than in BM (0.0%).

Discussion

A number of approaches to achieve allogeneic chimerism with lethal or nonlethal conditions have been reported in rodent models [3, 9-11]. Mixed allogeneic chimerism has several advantages over fully allogeneic chimerism. The presence of syngeneic (or autologous) BMCs appears to provide the necessary cells to overcome the impaired immunologic functions and prevent the GVHD observed in fully allogeneic chimeras [12], while the allogeneic BM elements appear responsible for the induction of donor-specific tolerance. In mixed chimeras, primary antibody responses to T cell-dependent antigens are completely restored, since T cells can cooperate with B cells and antigen-presenting cells. Therefore, mixed allogeneic chimerism may be a useful approach to induce tolerance for solid organ and cellular grafts.

Ildstad and *Sachs* were the first to establish a system of mixed allogeneic chimerism which can be used for organ transplantation [3]. Using skin allografts, they have shown that persistent tolerance can be induced until 380 days by carrying out mixed allogeneic BMT. In the present study using pancreas allografts, we have shown that persistent tolerance can be maintained for more than one year (480 days) after mixed allogeneic BMT, although the percentages of donor-type (C3H) cells gradually decrease (Fig. 1). This decrease in the percentages of donor (C3H)-type cells can be explained by MHC restriction between HSCs and

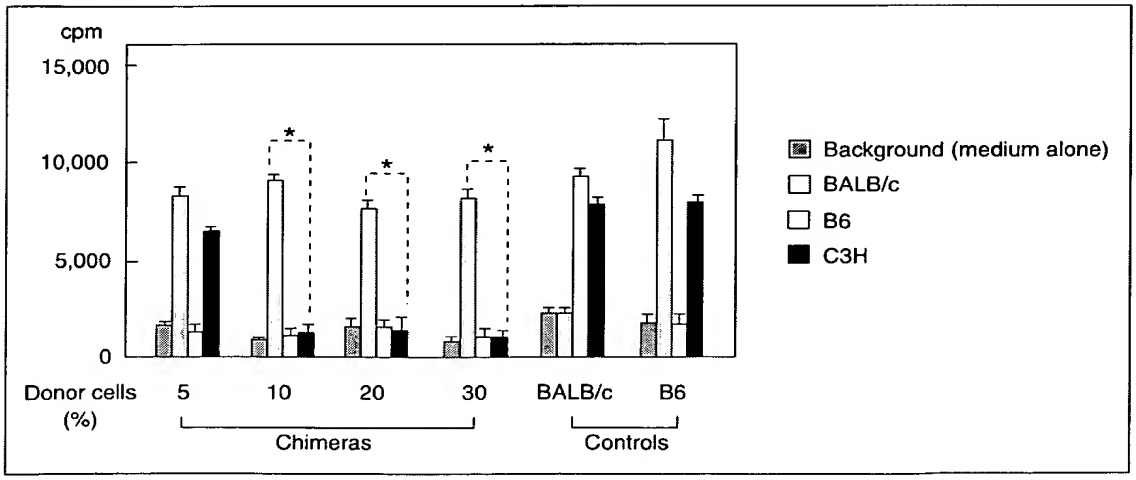


Figure 4. Relationship between percentages of donor cells and responsiveness in MLR six months after treatment. *The spleen cells of mixed chimeric mice with 5% of donor cells show a high responsiveness to donor (C3H)-type MHC determinants, although the spleen cells of mixed chimeric mice with more than an initial 10% of donor cells show low responsiveness to not only host-type but also donor-type MHC determinants. Asterisks represent* p *values of responses to donor-type stimulators versus third-party by* t*-test: *p < 0.001.*

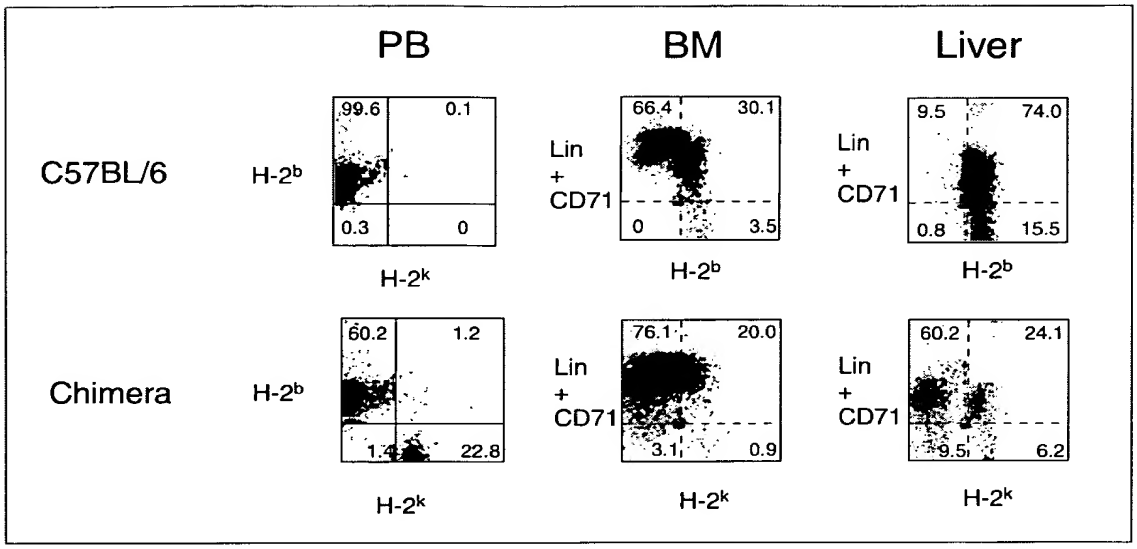


Figure 5. The characterization of the donor-derived BM and liver MNCs by flow cytometry three months after treatment. *BM and liver MNCs of chimeric mice and B/6 mice (control) were stained with H-2K^k, H-2K^b, CD71 mAbs, and lineage markers (CD4, CD8, B220, Mac-1, TER119, Gr-1). The populations of lineages CD71$^-$ and H-2K^{high} are HSC-enriched. Chimeric mice (four mice) were analyzed, and similar results were obtained. Therefore, representative data are shown in this figure.*

stromal cells; in the ([B6 + C3H]→B6) chimeric mice, B6 HSCs should show a better proliferative response than C3H HSCs, as we have previously shown [13]. It should be noted that all the pancreas allografts are accepted when more than 1% of allogeneic cells are detected in the PB of the recipients. To further examine the relationship between percentages of initially transplanted donor cells and acceptance of pancreatic allografts, we carried out mixed allogeneic BMT using donor BM of decreased percentages (5% to 30%). Table 2 shows that more than 10% of donor cells are necessary to prevent graft rejection. It has been reported that recipient mice with >30% chimerism accept skin allografts in an MHC class II-disparate combination, although recipients with <10% chimerism show prolonged skin graft survival but finally reject skins [14]. It is conceivable that the difference between the data of *Taniguchi et al.* and ours is due to the grafts of different organs (the skin and pancreas) and different mouse combinations (only class II-disparate combination in the former and both class I- and class II-disparate combinations in the latter).

In the present study, we have shown that pancreas allografts are accepted by the recipient mice with establishment of mixed chimerism in the PB despite marked decreases in donor-type cells (Figs. 1 and 2; Table 2). MLR (Fig. 3) and CTL (Table 1) assays indicate the induction of systemic tolerance in these mice. Mechanisms underlying tolerance induction include clonal deletion [15, 16], anergy [17], and suppression [18, 19]. In the present study, we have demonstrated the presence of donor-derived allogeneic HSCs in the BM and liver (Fig. 4). Since we have previously found that donor HSCs trapped in the liver induce clonal anergy in the recipient CD8$^+$ CTLs [20], it is certain that clonal anergy is involved in tolerance induction in this mixed allogeneic chimerism. Although we have not examined the clonal deletion mechanism in this system, it is also conceivable that clonal deletion, not only in the thymus but also in the periphery, is involved in this system, since *Zavazava et al.* have recently demonstrated that soluble MHC class I molecules induce apoptosis in alloreactive CTLs [21]. Suppressor mechanisms appear to be involved in the establishment of tolerance induction even in mixed allogeneic BMT. Suppressor cells include suppressor T cells (CD8$^+$ cells), natural killer cells, and natural suppressor (NS) cells; we have previously found that NS cells belong to HSCs in the cycling phase in the BM [22].

The skin and pancreas are candidates to examine functional tolerance in vivo, since they are highly antigeneic and very sensitive to rejection [7]. *Starzl et al.* have found in humans that there are some cases in which liver allografts survive without using immunosuppressants, and that, in such cases, a small number of donor-derived hemopoietic cells are detected. We have very recently established the method for organ allografts by injecting allogeneic hemopoietic cells (including HSCs) from the portal vein;

the recipient mice show microchimerism [17], as shown in the present study. It should be noted that the percentage of donor-type HSCs is higher in the liver (6.2%) than in the BM (0.9%) (Fig. 5). We have previously found that HSCs trapped in the liver induce anergy of recipient CTL2 [20]. We are in the process of analyzing the exact mechanisms underlying tolerance induction in microchimerism.

In summary, we report here that long-term pancreatic allograft survival over a one-year period can be reliably achieved in MHC-disparate allogeneic donor and recipient combinations, although donor-type cells gradually decrease. The tolerance was highly MHC-specific, as evidenced by MLR and CTL assays.

ACKNOWLEDGMENT

We thank *Ms. Keiko Ando* for preparing the manuscript.

This work was supported by a grant from the Japanese Ministry of Health and Welfare, the Ministry of Education, Science and Culture, Japan, and the Private School Promotion Foundation, Japan.

REFERENCES

1 Nakamura T, Good RA, Yasumizu R et al. Successful liver allografts in mice by combination with allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 1986;83:4529-4532.

2 Yasumizu R, Sugiura K, Iwai H et al. Treatment of type 1 diabetes mellitus in non-obese diabetic mice by transplantation of allogeneic bone marrow and pancreatic tissue. Proc Natl Acad Sci USA 1987;84:6555-6557.

3 Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. Nature 1984;307:168-170.

4 Starzl TE, Demetris AJ, Trucco M et al. Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. Hepatology 1993;17:1127-1152.

5 Hayashi H, Toki J, Lian Z et al. Analyses of extrathymic T cell differentiation in nu/nu mice by grafting embryonal organs. Immunobiology 1997;197:1-15.

6 Ogata H, Bradley WG, Inaba M et al. Long-term repopulation of hematolymphoid cells with only a few hematopoietic stem cells in mice. Proc Natl Acad Sci USA 1995;92:5945-5949.

7 Doi H, Inaba M, Yamamoto Y et al. Pluripotent hemopoietic stem cells are c-kit^{dow}. Proc Natl Acad Sci USA 1997;94:2513-2517.

8 Lian Z, Feng B, Sugiura K et al. c-kit^{dow} pluripotent hemopoietic stem cells form CFU-S on day 16. STEM CELLS 1999;17:39-44.

9 Li H, Colson YL, Ildstad ST. Mixed allogeneic chimerism achieved by lethal and nonlethal conditioning approaches induces donor-specific tolerance to simultaneous islet allografts. Transplantation 1995;60:523-529.

10 Li H, Kaufman CL, Boggs SS et al. Mixed allogeneic chimerism induced by a sublethal approach prevents autoimmune diabetes and reverses insulitis in nonobese diabetic (NOD) mice. J Immunol 1996;156:380-388.

11 Ildstad ST, Wren SM, Oh E et al. Mixed allogeneic reconstitution (A + B→A) to induce donor-specific transplantation tolerance. Permanent acceptance of a simultaneous donor skin graft. Transplantation 1991;51:1262-1267.

12 Ildstad ST, Wren SM, Bluestone JA et al. Effect of selective T cell depletion of host and/or donor bone marrow on lymphopoietic repopulation, tolerance, and graft-vs-host disease in mixed allogeneic chimeras (B10 + B10.D2→B10). J Immunol 1986;136:28-33.

13 Hashimoto F, Sugiura K, Inoue K et al. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. Blood 1997;89:49-54.

14 Taniguchi H, Abe M, Shirai T et al. Reconstitution ratio is critical for alloreactive T cell deletion and skin graft survival in mixed bone marrow chimeras. J Immunol 1995;155:5631-5636.

15 Yu JC, Webster M, Fox IJ. Clonal deletion: a mechanism of tolerance in mixed bone marrow chimeras. J Sur Res 1990;48:517-522.

16 Khan A, Tomita Y, Sykes M. Thymic dependence of loss of tolerance in mixed allogeneic bone marrow chimeras after depletion of donor antigen. Peripheral mechanisms do not contribute to maintenance of tolerance. Transplantation 1996;62:380-387.

17 Morita H, Sugiura K, Inaba M et al. A strategy for organ allografts without using immunosuppressants or irradiation. Proc Natl Acad Sci USA 1998;95:6947-6952.

18 Sykes M, Eisenthal A, Sachs DH. Mechanism of protection from graft-vs-host disease in murine mixed allogeneic chimeras. I. Development of a null cell population suppressive of cell-mediated lympholysis responses and derived from the syngeneic bone marrow component. J Immunol 1988;140:2903-2911.

19 Sykes M, Sachs DH. Mechanisms of suppression in mixed allogeneic chimeras. Transplantation 1988;46(suppl 2):135-142.

20 Sugiura K, Kato K, Hashimoto F et al. Induction of donor-specific T cell anergy by portal venous injection of allogeneic cells. Immunobiol 1997;197:460-477.

21 Zavazava N, Kronke M. Soluble HLA class I molecules induce apoptosis in alloreactive cytotoxic T lymphocytes. Nat Med 1996;2:1005-1010.

22 Sugiura K, Inaba M, Ogata H et al. Wheat germ agglutinin-positive cells in a stem cell-enriched fraction of mouse bone marrow have potent natural suppressor activity. Proc Natl Acad Sci USA 1988;85:4824-4826.

reference 2

0041-1337/02/7304-512/0
TRANSPLANTATION

Vol. 73, 512–518, No. 4, February 27, 2002
Printed in U.S.A.

TREATMENT OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS BY TRANSPLANTATION OF ISLET CELLS PLUS BONE MARROW CELLS VIA PORTAL VEIN IN RATS[1]

KAZUYA IKEBUKURO,[2,3] YASUSHI ADACHI,[2,4] YUICHIRO YAMADA,[5] SHIMPEI FUJIMOTO,[5] YUTAKA SEINO,[5] HARUKI OYAIZU,[2,6] KOSHIRO HIOKI,[3,4] AND SUSUMU IKEHARA[2,4,7]

First Department of Pathology, Second Department of Surgery, Transplantation Center, and First Department of Internal Medicine, Kansai Medical University, Osaka 570-8506, Japan; and Department of Metabolism and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

Background. **We have established a new method for the transplantation of allogeneic pancreatic islets (PIs) using sublethal irradiation (9 Gy) plus simultaneous transplantation of PIs and bone marrow cells (BMCs) via the portal vein (PV) followed by intravenous (i.v.) injection of donor BMCs (9 Gy + PV + i.v.).**

Methods. **Approximately 600 PIs of Brown Norway (BN: RT1A^n, RT1B^n) rats were transplanted into the liver of streptozotocin-induced diabetic Fischer 344 (F344: RT1A^l, RT1B^l) rats via the PV. BMCs (3×10^8) of BN rats were injected via the PV or i.v. into the recipients simultaneously. In some groups, additional i.v. injections of BMCs from BN rats were given 5 days after the PI transplantation.**

Results. **All the recipients (10 of 10) in the 9 Gy + PV + i.v. group showed normoglycemia for more than 1 year, whereas PIs were rejected within 30 days after transplantation in the group of 9 Gy + i.v. + i.v.**

Conclusions. **These results suggest that simultaneous transplantation of PIs and BMCs via the PV is effective in inducing persistent tolerance.**

Insulin-dependent diabetes mellitus (IDDM) is mainly juvenile-onset, nonobese, and ketosis-prone diabetes. In IDDM, insulin-producing beta cells of the pancreatic islets (PIs) are progressively destroyed, insulin production is reduced, and the plasma insulin level becomes extremely low (*1*). Exogenous insulin injection has prolonged the survival of diabetic patients and has prevented systemic complications (*2*). However, it is difficult to tightly control glucose homeostasis using insulin injections. The transplantation of isolated PIs is a physiologic approach to the replacement of pancreatic endocrine functions, and is more easily performed than whole pancreas transplantation. It has been recognized that transplanted isolated PIs are functional as a regulator of blood glucose (*3*, *4*). Transplanted PI grafts with portal venous drainage are more efficient in normalizing glucose metabolism than systemically draining PI grafts (*5–8*). Although improved immunosuppressive agents and techniques for the isolation of large numbers of functional PIs have made PI transplantation a possible therapeutic approach for diabetic patients, rejection remains a major limitation preventing widespread clinical application.

It is well known that successful transplantation of allogeneic PIs is difficult because of their strong antigenicity (*9*). There are many problems in the allogeneic transplantation of PIs, a major one being the development of acute and chronic rejection despite continuous usage of immunosuppressants. In addition, side effects of continuous immunosuppression are reported: these include the development of malignancies, opportunistic infections, and organ toxicity. The first association between bone marrow chimerism and tolerance was reported in 1953 (*10*). The donor-specific tolerance induced by bone marrow chimerism was found to eliminate the problem of allograft rejection (*11–15*).

It is also well known that the portal venous administration of alloantigens can induce tolerance. Callery et al. (*16*) and Genden et al. (*17*) have reported that the administration of donor cells via the portal vein (PV) promotes peripheral donor-specific hyporesponsiveness and prevents allografts of organs and tissues.

We have previously found that the administration of allogeneic cells via the PV induces donor-specific tolerance across MHC barriers (*18*), and that donor hemopoietic stem cells (HSCs), which are trapped in the liver after PV injection, induce anergy to host CD8$^+$ T cells owing to the absence of costimulatory signals (*19*). We have also found that the injection of HSCs via the PV plus short-term administration of an immunosuppressant (cyclosporine or FK-506) can induce persistent tolerance in the skin allografts in mice (*20*) and pigs (*21*).

In the present study, we show a strategy to allow the long-term acceptance of allogeneic PIs with normal functions without recourse to the use of immunosuppressants: 9 Gy irradiation and intrahepatic transplantation of PIs plus PV injection of whole donor bone marrow cells (BMCs), followed by i.v. injection of whole donor BMCs 5 days after PI transplantation.

[1] This work was supported by a grant from the Haiteku Research Center of the Ministry of Education, a grant from the Millennium program of the Ministry of Education, Culture, Sports, Science and Technology, a grant from the Science Frontier program of the Ministry of Education, Culture, Sports, Science and Technology, grant-in-aid for scientific research (B) 11470062, grants-in-aid for scientific research on priority areas (A)10181225 and (A)11162221, and also a grant from Japan Immunoresearch Laboratories Co., Ltd. (JIMRO).

[2] First Department of Pathology, Kansai Medical University.

[3] Second Department of Surgery, Kansai Medical University.

[4] Transplantation Center, Kansai Medical University.

[5] Department of Metabolism and Clinical Nutrition, Graduate School of Medicine, Kyoto University.

[6] First Department of Internal Medicine, Kansai Medical University.

[7] Address correspondence to: Susumu Ikehara, MD, PhD, First Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi City, Osaka 570-8506, Japan. E-mail: ikehara@takii.kmu.ac.jp

MATERIALS AND METHODS

Animals

Eight- to 10-wk-old male Brown Norway (BN, RT1A^n, RT1B^n) and 7- to 9-wk-old female Fischer 344 (F344, RT1A^l, RT1B^l) rats were purchased from Clea Japan Inc. (Tokyo, Japan) as donors and recipients, respectively, and maintained in our conventional animal facilities.

Pharmacologic Induction of Diabetes

Diabetes mellitus was induced in normal F344 rats by a single i.p. injection of 70 mg/kg of streptozotocin (Nacalai Tesque, Osaka, Japan), which is directly toxic to beta cells and induces an outbreak of clinical diabetes within 3 days (*22*). Diabetic F344 rats that showed more than 400 mg/dl for at least 3 consecutive days in nonfasting plasma glucose were used as recipients.

Transplantation

PIs were isolated by the collagenase technique as previously described (*23*). Diabetic F344 rats were irradiated (8, 8.5, or 9 Gy) with a single dose from a ^{137}Cs source 1 day before transplantation. Under pentobarbital (Essex Animal Health Friesoythe, Friesoythe, Germany) anesthesia, the peritoneal cavity of the recipient was accessed through a midline incision. The 600 PIs from BN rats were transplanted into the liver via the PV using a 26-gauge needle. After infusion, the syringe was rinsed several times by repeated aspiration and reinfusion of PV blood. BMCs were collected from the femurs and tibias of BN rats, and 3×10^8 whole BMCs were injected intraportally (PV) or i.v. into the recipients simultaneously. In some groups, additional i.v. injections of BMCs from BN rats were given via the lateral tail vein 5 days after the PI transplantation. Several experimental groups were set up: (1) 9 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs after 5 days (9 Gy+PV+i.v., n=10), (2) 9 Gy irradiation plus PV injection of PIs and i.v. injection of BMCs followed by i.v. injection of BMCs after 5 days (9 Gy+i.v.+i.v., n=7), (3) 9 Gy irradiation plus PV injection of PIs and BMCs (9 Gy+PV; n=10), (4) 8.5 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs after 5 days (8.5 Gy+PV+i.v., n=9), (5) 8.5 Gy irradiation plus PV injection of PIs and i.v. injection of BMCs followed by i.v. injection of BMCs after 5 days (8.5 Gy+i.v.+i.v., n=7), (6) 8.5 Gy irradiation plus PV injection of PIs and BMCs (8.5 Gy+PV; n=7), and (7) 8 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs (8 Gy+PV+i.v., n=8). The graft-accepting recipients showed no morbidity (diarrhea, cachexia, or alopecia, etc.) after transplantation.

Normal F344 rats did not die after 9 Gy irradiation because of the recovery of their own BMCs even when no BMCs were injected (data not shown). Therefore, we conclude that 9 Gy is a sublethal irradiation dose for rats.

Criteria for Rejection

Graft rejection was considered to have occurred when posttransplantation nonfasting plasma glucose levels exceeded 300 mg/dl for two consecutive measurements.

Flow Cytometric Analyses

MHC class I (RT1A^l or RT1A^n) surface markers were examined for the peripheral blood mononuclear cells (PBMCs) of recipients on the days after transplantation indicated in Figure 3 by a FACScan (Becton Dickinson, Mountain View, CA). Briefly, peripheral blood was layered over lymphocyte separation medium (Lympholyte-Mammal, Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) and centrifuged at 18°C (5000 rpm for 30 min). The lymphocyte layer was aspirated from the serum-lymphocyte separation medium interface and washed. Cells were stained with fluorescein isothiocyanate-labeled anti-RT1A^l monoclonal antibodies (mAbs) against recipient MHC class I (RT1A^l, PharMingen, San Diego, CA), and mAbs against donor MHC class I (RT1A^n, Serotec, Oxford, England), followed by staining with phycoerythrin-labeled anti-mouse IgG Abs (Serotec).

Glucose Tolerance Tests

Five months after the PI transplantation, glucose tolerance tests (GTTs) were performed. Food was removed for 16 hr before the GTTs were started. The rats in each experimental group were injected i.v. with 2 mg of glucose per gram of body weight. Blood samples were obtained 15, 30, 60, and 120 min after the glucose injection. Plasma glucose levels were analyzed by a glucose oxidase method.

Serum Insulin Measurements

Food was removed for 16 hr before the serum insulin levels were measured. Rats were injected i.v. with 2 mg of glucose per gram of body weight. Blood samples were obtained at 30 min after glucose injection and measured for serum insulin levels using an ELISA method (*23*).

Histologic Findings

The livers of the recipient rats were removed and fixed in 10% buffered formalin, processed for light microscopy, and stained with hematoxylin and eosin (H-E) and with anti-insulin antibody to identify the grafted PIs.

Mixed Lymphocyte Reaction

For mixed lymphocyte reaction (MLR), splenic T cells were obtained by passing spleen cells through a nylon-wool fiber (Wako Pure Chemical, Osaka, Japan) column after incubation for 60 min at 37°C; 1 g of the nylon-wool fiber was prepared for 3×10^7 splenocytes to use as responders. Lymphocytes (3×10^6) were cocultured with 20 Gy-irradiated whole spleen cells (3×10^6: stimulator cells) in a total of 200 μl of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 50 μM of 2-mercaptoethanol (2-ME; Wako). The cultures were incubated at 37°C for 5 days in a humidified 5% CO_2 environment in 96-well flat-bottom microwell trays (Corning Glass Works, Corning, NY), and pulsed with 0.5 μCi [^{3}H]-thymidine per well for the last 20 hr of the culture period. Stimulation indices were calculated by normalization to self-reactivity, which was near background incorporation in all cases.

Assay for Generation of Cytotoxic T Lymphocytes

Cytotoxic T-lymphocyte (CTL) assays were evaluated by measuring the release of lactate dehydrogenase (LDH) from target cells using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kits (Promega, Madison, WI), following the manufacturer's instructions. Briefly, splenocytes from F344 (RT1A^l), BN (RT1A^n), or ACI (August Copenhagen Irish; RT1A^a) rats were cultured with 2.5 mg/ml concanavalin A (Con A, Sigma, St. Louis, MO) for 3 days to prepare the Con A blastocytes as target cells. After washing, 3×10^5/well of Con A blastocytes were incubated for another 12 hr with or without 1×10^6/well of splenocytes from posttransplantation F344 or BN rats.

Statistical Analyses

Statistical analyses were performed using a two-tailed Student's *t*-test, except for graft survival rates. Statistical analyses of graft survival rates were performed using a log rank test.

RESULTS

Graft Survival

Because we have found that the injections of BMCs via the PV plus i.v. are effective in inducing persistent tolerance not only in chimeric-resistant MRL/lpr mice (*24*) but also in skin allografts of mice (*20*, *25*) and pigs (*21*), we have attempted to examine the effect of PV injection of BMCs on tolerance induction using the PI transplantation system. To detect the

diabetic condition, nonfasting blood glucose levels were monitored every other day in F344 rats in which diabetes mellitus had been induced by streptozotocin. Plasma glucose levels in all recipient rats returned to normal within 24 hr after the PI transplantation (Fig. 1A). We monitored the graft survival by measuring nonfasting blood glucose levels. In the 8 Gy+PV+i.v., 8.5 Gy+i.v.+i.v., and 9 Gy+i.v.+i.v. groups, all grafts were rejected within 30 days. The 9 Gy+PV group showed a 70% graft survival rate 320 days after transplantation, whereas the 9 Gy+PV+i.v. group showed a 100% graft survived rate more than 1 year after the PI transplantation. All rats (10 of 10) in the 9 Gy+PV+i.v. group showed normoglycemia for more than 1 year. The graft survival rate in the 8.5 Gy+PV+i.v. group was 44% 150 days after transplantation (Fig. 1). In the 8.5 Gy+PV group, only one in seven cases accepted the PIs until day 185. These results suggest that simultaneous PV injection of BMCs induces tolerance to allogeneic PIs more easily than the i.v. injection of BMCs, and that the additional i.v. injection of BMCs maintains the tolerance induced by the PV injection of BMCs.

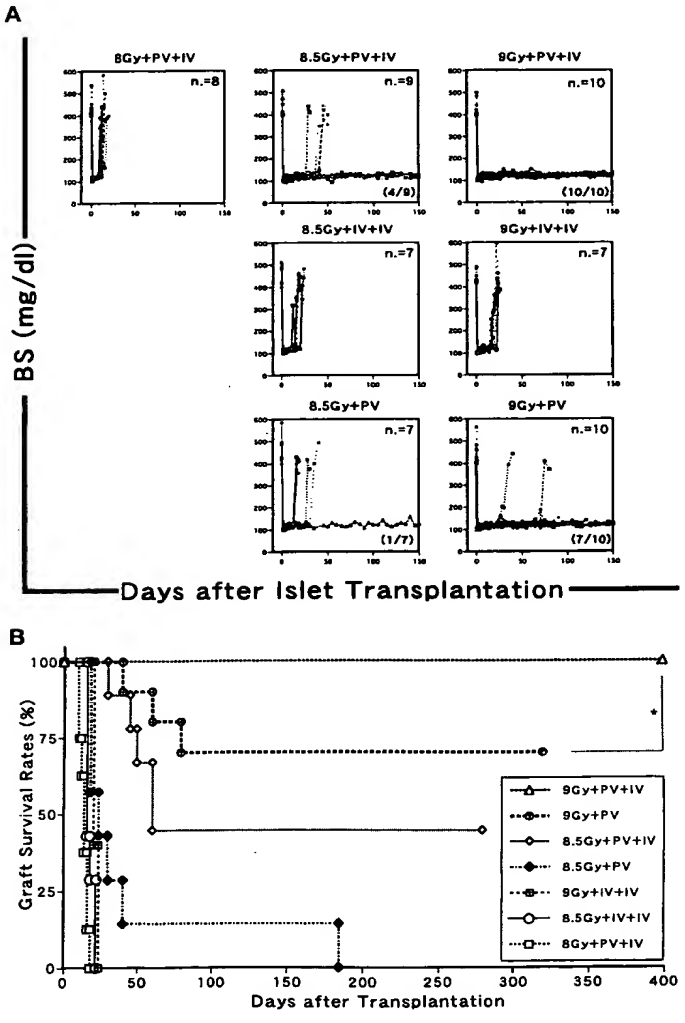


FIGURE 1. Graft survival of transplanted PIs. After irradiation, the PIs of BN rats were transplanted into F344 rats with BMCs of BN rats via the PV or i.v. Additional i.v. injection of BMCs from BN rats was performed in some groups, as described in *Materials and Methods*. Graft survival terms (A) and graft survival rates (B) in each experimental group are shown (*$P<0.01$). BS, blood sugar.

Histologic Findings

The livers of recipient rats were stained with H-E and with anti-insulin antibody to identify the grafted PIs. Grafted PIs were found in the 9 Gy+PV+i.v. group (60 days after transplantation; Fig. 2A), whereas disrupted islets, into which lymphocytes and macrophages had infiltrated, were seen in the livers of the 9 Gy+i.v.+i.v. group 30 days after transplantation (Fig. 2B). In the livers of the 9 Gy+PV+i.v. group, the grafted PIs were found even 200 days after the transplantation (Fig. 2C), although they were scattered throughout the liver in contrast with their distribution at 60 days after transplantation. The PIs grafted via the PV formed clusters in the liver for a short time, but they later became scattered throughout the liver. This seems to be related to the fact that the hepatocytes can proliferate, while the PI cells cannot.

Insulin was positively stained in the grafted PIs (Fig. 2D). In contrast, infiltrating mononuclear cells and fibrosis were seen in the livers of the 9 Gy+i.v.+i.v. group, and no insulin-positive PIs were found in the livers (data not shown). These results suggest that functional PIs exist in the livers of normoglycemic recipient rats, and that the grafted PIs are destroyed by immunologic mechanisms in the livers of hyperglycemic recipient rats.

Flow Cytometric Analyses of PBMCs

Chimerism was evaluated by flow cytometric analyses 90 days after transplantation using recipient PBMCs and anti-class I rat mAbs. The PBMCs in all graft-accepting rats showed the donor phenotype, although those of one graft-accepting rat in the 8.5 Gy+PV group partially showed the recipient phenotype, the level being 18.4% on day 90. Moreover, the transplanted PIs of this rat were rejected on day 185 when PBMCs showed only the recipient phenotype. The PBMCs in all the graft-rejecting rats showed the recipient phenotype (Fig. 3). However, all the PI-transplanted rats treated with 9 Gy+PV+i.v. survived more than 1 year, and their PBMCs were donor-type cells (>98%; Fig. 3). These results suggest that the existence of donor bone marrow-derived cells is crucial for successful transplantation of the PIs.

Intravenous Glucose Tolerance Tests and Serum Insulin Levels. As nonfasting blood glucose levels do not provide an accurate assessment of true regulation of glucose homeostasis in the PI graft-accepting recipients, i.v. glucose tolerance tests (IVGTTs) were performed and the serum insulin levels measured to evaluate the functions of the grafted PIs 150 days after transplantation. We compared the IVGTTs between the rats treated with 9 Gy+PV+i.v. and untreated control F344 rats (Fig. 4A). Fasting blood glucose levels were not significantly different between the graft-accepting rats (n=6) and untreated normal control rats (n=6). At 15 and 30 min after glucose administration, the PI-accepting rats showed a slightly higher blood glucose level than untreated normal control rats, although there was no significant difference. At 60 and 120 min after glucose administration, the blood glucose levels in the PI-accepting rats and untreated normal control rats returned to the levels before glucose administration and showed similar values.

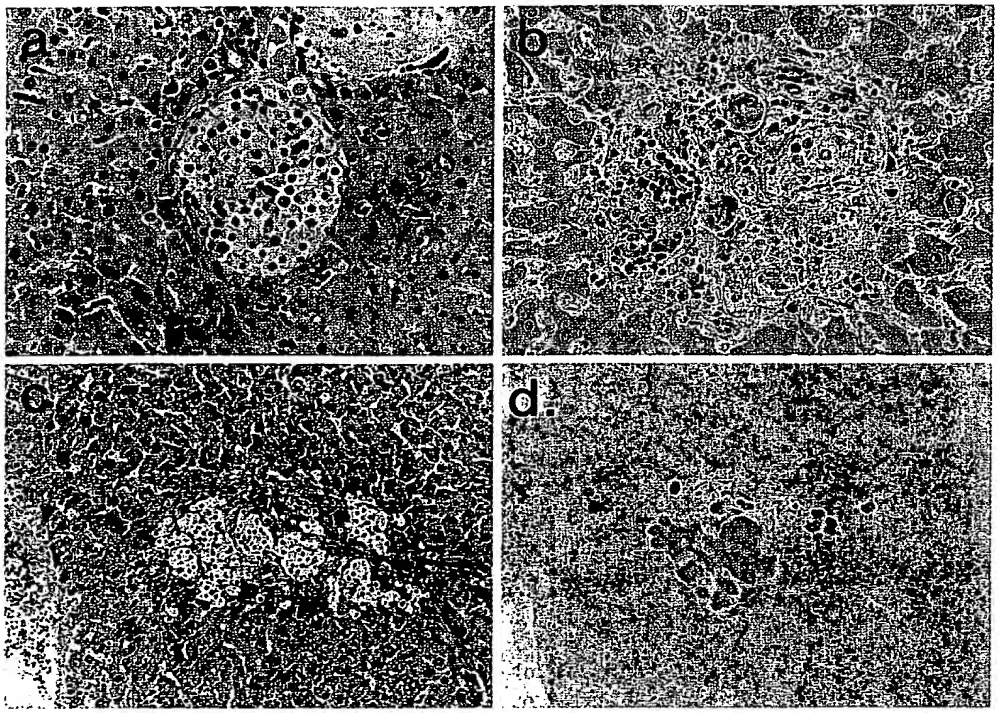


FIGURE 2. Histologic findings of transplanted PIs. PI-transplanted rats were killed 60 days after transplantation, followed by the histologic study of the livers, into which allogeneic PIs had been transplanted via the PV. Histologic examination was performed as described in *Materials and Methods*. The liver of a PI-transplanted rat with normoglycemia in nonfasting blood (a, H-E staining) and the liver of a PI-transplanted rat with hyperglycemia (b, H-E staining) are shown. PI-transplanted rats with normoglycemia were killed also 180 days after transplantation, and representative data on H-E staining (c) or immunohistologic staining for insulin (d) are shown.

We next measured the serum insulin levels in the groups of untreated normal control F344 rats (n=7), diabetes mellitus (DM)-induced nongrafting rats (n=7), PI-rejecting rats (n=7), and PI-accepting rats (n=12; Fig. 4B). The insulin levels in the group of DM-induced and PI-rejecting rats were significantly lower than those in the group of untreated normal rats. The insulin levels in PI-accepting rats were significantly higher than those in the PI-rejecting rats and were slightly lower than those in untreated normal rats, although there was no significant difference. These results suggest that the insulin levels of the DM rats do not recover owing to the destruction of beta cells, and that glucose homeostasis is maintained by the grafted PIs. The insulin levels in the rats treated with 9 Gy irradiation plus PV injection of only BMCs (without grafting PIs) followed by i.v. injection of BMCs after 5 days were similar to those of DM-induced rats (data not shown).

Mixed Lymphocyte Reaction

The splenic T cells of recipient rats that had received the PIs and BMCs from the donors were examined for their reactivity to the recipient, donor, and third-party alloanti-

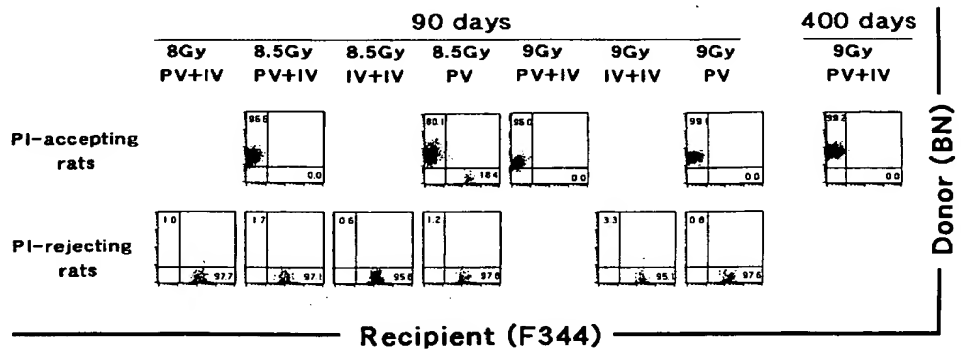


FIGURE 3. Fluorescence-activated cell sorter analyses using PBMCs of posttransplanted rats. The phenotypes of MHC class I in the PBMCs obtained from the PI-transplanted rats 90 days after various treatments (as indicated in this figure) or 400 days after the treatment with PV+i.v. were analyzed using a FACScan. Representative data in each experimental group are shown.

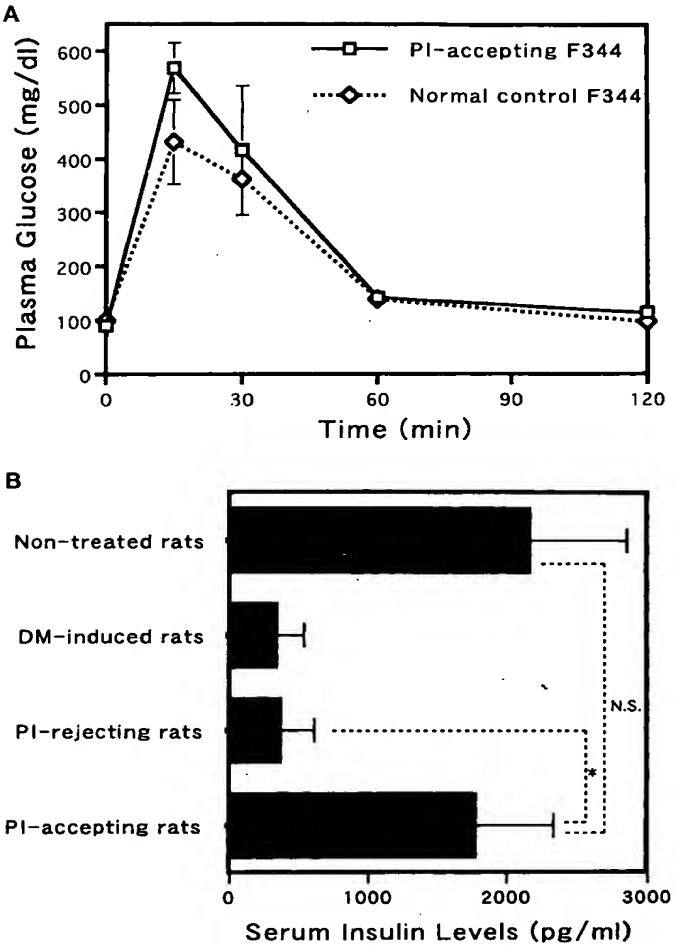


FIGURE 4. **(A) IVGTTs of PI-transplanted rats in the 9 Gy+PV+i.v. group with normoglycemia in nonfasting blood were performed, and compared with those of untreated normal control rats. (B) Normal serum insulin levels of PI-accepting rats. Serum insulin levels were measured 150 days after PI transplantation, as described in *Materials and Methods* (*$P<0.005$, N.S., not significant).**

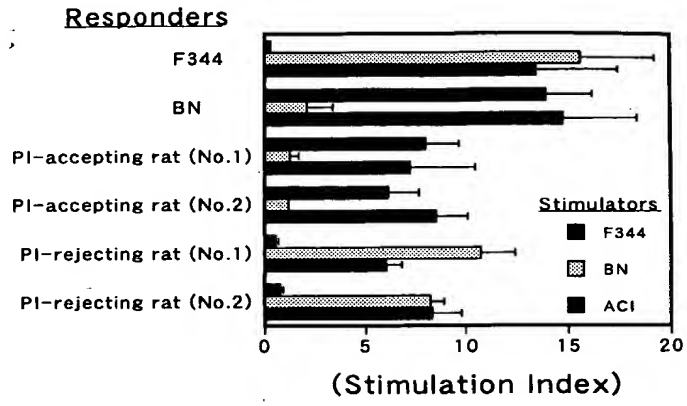


FIGURE 5. **MLR in T cells from posttransplanted rats. MLR assays were performed as described in *Materials and Methods*. Splenocytes (3×10^7) as stimulators from untreated F344, BN, and ACI rats were cultured for 5 days with or without T cells (3×10^6) of posttransplanted F344, BN, or ACI rats. They were pulsed with 0.5 μCi [^{3}H]-thymidine per well for the last 20 hr of the culturing period. Stimulation indices were calculated by normalization to self-reactivity, which was near background incorporation in all cases.**

gens using MLR assays 150 days after transplantation (Fig. 5). Untreated normal control F344 and BN rats showed normal proliferative responses to stimulator cells. The T cells of the graft-rejecting rats showed no response to the splenic cells of recipient F344 rats, although they did show responses to both donor and third-party MHC determinants. In contrast, the T cells of graft-accepting recipients, which were tolerant to donor alloantigens, showed similar proliferative responses to recipient stimulator cells as well as MHC-disparate third-party rat stimulator cells. Thus, the T cells of the rats with successful transplants responded to not only third-party cells but also host-type cells, although the graft-accepting recipients did not show any symptoms of graft-versus-host reaction (GvHR) for more than 1 year.

CTL Assays

Inasmuch as the graft-accepting rats showed the proliferative responses to host splenocytes in MLR assays but showed no symptoms of GvHR, we next performed the assays for the generation of CTLs to examine whether transplanted HSCs show cytotoxic effects on recipient cells in vitro. As shown in Figure 6, the splenocytes of F344 rats showed cytotoxic effects on both BN rat and third-party ACI rat cells, and the splenocytes of the donor BN rats showed cytotoxic effects on the recipient F344 rat cells. However, the graft-accepting rats showed no cytotoxic effects on not only donor rat (BN) cells but also recipient rat (F344) cells, although they showed a significant response to ACI (a third-party) rat cells. These results suggest that the transplanted donor HSCs do not injure the host cells even in vitro, although the T cells of the graft-accepting rats showed the proliferative responses to host cells in MLR assays.

DISCUSSION

Successful transplantation of tissues, cells, and organs between fully MHC-mismatched donor and recipient combinations has been dependent on the use of immunosuppressive agents to control acute and chronic rejection. Immunosuppressants have some toxic effects on various cells, which results in significant morbidity and mortality. It has been recognized that the induction of donor-specific tolerance by bone marrow chimerism can eliminate the problem of allograft rejection (*11–15*). Although bone marrow chimerism can successfully prevent even chronic graft rejection, the clinical application of the lethal conditioning approach to induce tolerance would be limited by the excessive toxicity associated with lethal conditioning.

The administration of insulin for the treatment of IDDM usually controls blood glucose and prevents lethal diabetic ketoacidosis. However, even frequent insulin treatments are not enough to prevent the high morbidity and mortality associated with IDDM (*26*). It has been reported that the transplantation of whole pancreas can maintain normal blood glucose levels and effectively control IDDM (*27*). The transplantation of the endocrine tissue alone (isolated PIs) is a

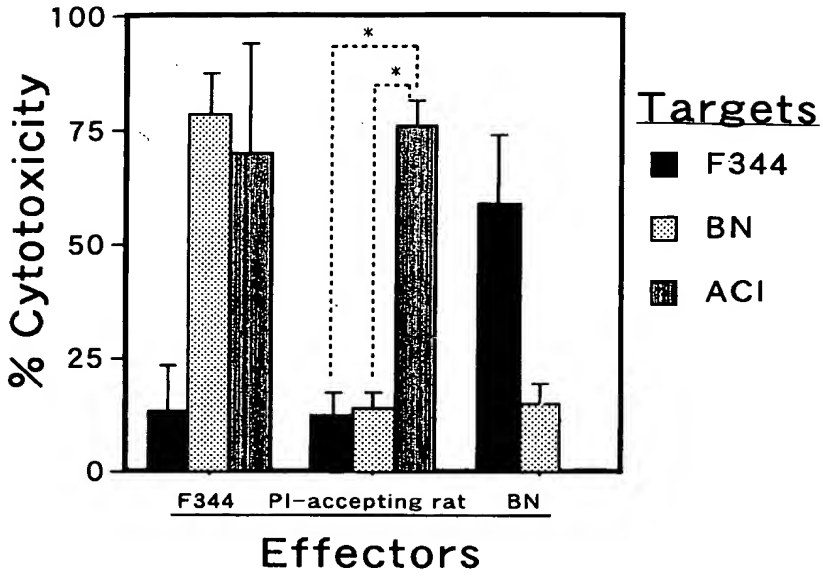


FIGURE 6. CTL assays. Con A blastocytes (3×10^5), which were prepared from splenocytes (1×10^6) of F344, BN, and ACI rats, were cultured for 12 hr with or without splenocytes from posttransplanted F344 or ACI rats, followed by measuring the levels of LDH in the wells using CytoTox 96 (*$P<0.05$).

more technically simple approach than whole pancreatic transplantation. It has also been reported that the survival term of the grafts in the transplantation of PIs is short owing to their high antigenicity and their high sensitivity to graft rejection (*28*).

It has been reported that systemically draining grafts (such as PI grafts under the renal capsule) directly secrete insulin into the systemic circulation and induce hyperinsulinemia (*29*). Therefore, the transplantation of PIs via the PV (PV drainage) is a more physiologic approach than systemic drainage (*5, 8*), and intrahepatic PI transplantation is easily performed. Clinically, PV injection is easily carried out under echographically guided portal puncture with local anesthesia. We have previously found that the PV administration of allogeneic cells can induce donor-specific tolerance across MHC barriers (*18*), and that the injection of BMCs via the PV followed by transient usage of an immunosuppressant without irradiation can induce persistent tolerance in skin allografts (*20, 21*). In the present study, we have found a strategy for long-term acceptance of PIs with normal functions without recourse to immunosuppressants: a sublethal irradiation dose (9 Gy) plus simultaneous injection of BMCs and PIs via the PV followed by i.v. injection of BMCs. The 9 Gy+PV+i.v. group showed normal glucose levels and a 100% survival rate, and did not develop IDDM by 400 days after transplantation (Fig. 1), whereas the 9 Gy+i.v.+i.v. group showed high glucose levels owing to the rejection of the PIs (Fig. 1A). The PBMCs in all but one graft-accepting rat showed the donor phenotype. The exception was in the 8.5 Gy+PV group; 18.4% recipient phenotype was detected in the PBMCs in this rat at 90 days after transplantation, indicating that mixed chimerism existed at that time. The PI graft of this rat was rejected 185 days after transplantation, at which time no donor cells were detected in the PBMCs. The mechanism underlying the loss of mixed chimerism has not been elucidated, but the loss may reflect the presence of MHC restriction (preference) between HSCs and stromal cells, as we previously described (*30, 31*). Therefore, the complete replacement of hemopoietic cells by donor cells (instead of mixed chimeras) is crucial to the induction of persistent tolerance, as we have previously shown in lethally irradiated mice (*1, 11–13, 24*). We have previously used T-cell–depleted BMCs for allogeneic bone marrow transplantation (BMT). However, we have recently used whole BMCs, which contain a small number of T cells (<1%). The T cells present in the bone marrow were found not to induce GvHR, but to facilitate bone marrow engraftment (prevent HvGR) (*32, 33*), even when sublethal irradiation doses were used for allogeneic BMT (*34*) and organ transplantation (*25*). It should be noted that sublethal irradiation (7 Gy for mice (*25*) and 9 Gy for rats in the present study) induces fully allogeneic chimerism (>98%) for more than 1 year without evidence of either GvHR or HvGR.

It is thought that IDDM is an organ-specific autoimmune disease, which is characterized by the destruction of insulin-producing beta cells by autoimmune mechanisms (*1*). The nonobese diabetic (NOD) mouse is a well-known animal model for IDDM. We have previously shown that allogeneic BMT can prevent and treat insulitis (*1*), and that allogeneic BMT plus fetal pancreas grafts can treat overt diabetes in NOD mice (*12*). However, we have found that in (BALB/c+NOD→NOD) chimeric mice, NOD hemopoietic cells become dominant, which results in the development of IDDM (*35*), because the abnormal HSCs of autoimmune-prone mice are more resilient than normal HSCs, as we previously described (*36*). These findings suggest that allogeneic BMT instead of mixed allogeneic BMT should be carried out in conjunction with organ transplantation.

Hemopoietic cells from PI-accepting rats showed the proliferative response to host cells in MLR assays in vitro. However, they showed no cytotoxic effects on host cells not only in vivo (no GVHR) but also in CTL assays in vitro. This split tolerance, as previously described by Sprent et al. (*37*), is interesting for analyzing the mechanisms underlying tolerance induction. It is conceivable that some suppressor mechanisms that inhibit the functions of cytotoxic effector cells

against the host are involved. We are now in the process of examining regulatory and suppressor cells in our system.

In conclusion, we have demonstrated that the combination of PV plus i.v. injections of BMCs is effective in inducing donor-specific tolerance across MHC barriers, even when the irradiation dose is reduced to 9 Gy (sublethal dose). This strategy (9 Gy+PV+i.v.) leads to long-term acceptance of PIs with normal functions, enabling the treatment of diabetes mellitus without recourse to immunosuppressants.

Acknowledgments. The authors thank Y. Tokuyama, M. Shinkawa, and S. Miura for their expert technical assistance, and Hilary Eastwick-Field and K. Ando for their help in the preparation of the manuscript.

REFERENCES

1. Ikehara S, Ohtsuki H, Good RA, et al. Prevention of type 1 diabetes in nonobese diabetic mice by allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 1985; 82: 7743.
2. Castano L, Eisenbarth GS. Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. Annu Rev Immunol 1990; 8: 647.
3. Wilson JD, Prowse SJ, Haynes SP. Pancreatic islet allograft function in nonimmunosuppressed conscious mice. Metabolism 1985; 34: 92.
4. Miriam A. Metabolic and morphologic studies in intraportal-islet-transplanted rats. Diabetes 1976; 25: 1041.
5. Gores PF, Rabe F, Sutherland DE. Prolonged survival of intraportal versus subrenal capsular transplanted islet allografts. Transplantation 1987; 43: 747.
6. Cuthbertson RA, Mandel TE. A comparison of portal versus systemic venous drainage in murine foetal pancreatic islet transplantation. Australian J Exp Biol Med Sci 1986; 64: 175.
7. Gilles MC, Mandel TE. The evolution of function and response to arginine challenge and pregnancy of portally and systemically placed islet cell grafts in streptozotocin diabetic mice. Metabolism 1990; 39: 1253.
8. Brown J, Mullen Y, Clark WR, Molner IG, Heininger D. Importance of hepatic portal circulation for insulin action in streptozotocin-diabetic rats transplanted with fetal pancreas. J Clin Invest 1979; 64: 1688.
9. Zeng Y, Ricordi C, Tzakis A, et al. Long-term survival of donor-specific pancreatic islet xenografts in fully xenogeneic chimeras. Transplantation 1992; 53: 277.
10. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature 1953; 172: 603.
11. Nakamura T, Good RA, Yasumizu R, et al. Successful liver allografts in mice by combination with allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 1986; 83: 4529.
12. Yasumizu R, Sugiura K, Iwai H, et al. Treatment of type 1 diabetes mellitus in non-obese diabetic mice by transplantation of allogeneic bone marrow and pancreatic tissue. Proc Natl Acad Sci USA 1987; 84: 6555.
13. Iwai H, Yasumizu R, Sugiura K, et al. Successful pancreatic allografts in combination with bone marrow transplantation in mice. Immunology 1987; 62: 457.
14. Exner BG, Fowler K, Ildstad ST. Tolerance induction for islet transplantation. Ann Transplant 1997; 2: 77.
15. Neipp M, Exner BG, Ildstad ST. A nonlethal conditioning approach to achieve engraftment of xenogeneic rat bone marrow in mice and to induce donor-specific tolerance. Transplantation 1998; 66: 969.
16. Callery MP, Kamei T, Flye MW. Kupffer cell blockade inhibits induction of tolerance by the portal venous route. Transplantation 1989; 47: 1092.
17. Genden EM, Mackinnon SE, Yu S, Flye MW. Induction of donor-specific tolerance to rat nerve allografts with portal venous donor alloantigen and anti-ICAM-1/LFA-1 monoclonal antibodies. Surgery 1998; 124: 448.
18. Zhang Y, Yasumizu R, Sugiura K, et al. Fate of allogeneic or syngeneic cells in intravenous or portal vein injection: possible explanation for the mechanism of tolerance induction by portal vein injection. Eur J Immunol 1994; 24: 1558.
19. Sugiura K, Kato K, Hashimoto F, et al. Induction of donor-specific T cell anergy by portal venous injection of allogeneic cells. Immunobiology 1997; 197: 460.
20. Morita H, Sugiura K, Inaba M, et al. A strategy for organ allografts without using immunosuppressants or irradiation. Proc Natl Acad Sci USA 1998; 95: 6947.
21. Morita H, Nakamura N, Sugiura K, et al. Acceptance of skin allografts in pigs by portal venous injection of donor bone marrow cells. Ann Surg 1999; 23: 114.
22. Elias D, Prigozin H, Polak N, Rapoport M, Lohse AW, Cohen IR. Autoimmune diabetes induced by the β-cell toxin STZ. Diabetes 1994; 43: 992.
23. Miyawaki K, Yamada Y, Yano H, et al. Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. Proc Natl Acad Sci USA 1999; 96: 14843.
24. Kushida T, Inaba M, Takeuchi K, Sugiura K, Ogawa R, Ikehara S. Treatment of intractable autoimmune diseases in MRL/lpr mice using a new strategy for allogeneic bone marrow transplantation. Blood 2000; 95: 1862.
25. Jin T, Toki J, Inaba M, et al. A novel strategy for organ allografts using sublethal (7Gy) irradiation followed by injection of donor bone marrow cells via portal vein. Transplantation, in press.
26. Smith RM, Mandel TE. Pancreatic islet xenotransplantation: the potential for tolerance induction. Immunol. Today 2000; 21: 42.
27. Gruessner RW, Sutherland DE, Najarian JS, Dunn DL, Gruessner AC. Solitary pancreas transplantation for nonuremic patients with labile insulin-dependent diabetes mellitus. Transplantation 1997; 64: 1572.
28. Li H, Colson YL, Ildstad ST. Mixed allogeneic chimerism achieved by lethal and nonlethal conditioning approaches induces donor-specific tolerance to simultaneous islet allografts. Transplantation 1995; 6: 523.
29. Nymann T, Shokouh-Amiri MH, Elmer DS, Stratta RJ, Gaber AO. Diagnosis, management, and outcome of late duodenal complications in portal-enteric pancreas transplantation. J Am Coll Surg 1997; 185: 560.
30. Hashimoto F, Sugiura K, Inoue K, Ikehara S. Major histocompatibility complex restriction between hematopoietic stem cells and stromal cells in vivo. Blood 1997; 89: 49.
31. Hayashi H, Toki J, Lian Z, Inoue K, Ikehara S. Analyses of extrathymic T cell differentiation in nu/nu mice by grafting embryonal organs. Immunobiology 1997; 197: 1.
32. Gandy KL, Domen J, Aguila H, Weissman IL. $CD8^+TCR^+$ and CD^+TCR^- cells in whole bone marrow facilitate the engraftment of hematopoietic stem cells across allogeneic barriers. Immunity 1999; 11: 579.
33. Takeuchi K, Inaba M, Miyashima S, Ogawa R, Ikehara S. A new strategy for treatment of autoimmune diseases in chimeric resistant MRL/lpr mice. Blood 1998; 91: 4616.
34. Kushida T, Inaba M, Hisha H, et al. Intra-bone marrow injection of allogeneic bone marrow cells: a powerful new strategy for treatment of intractable autoimmune diseases in MRL/lpr mice. Blood 2001; 97: 3292.
35. Takao F, Yasumizu R, Soe T, et al. Development of insulin-dependent diabetes mellitus in [(NOD + BALB/c) → NOD] mixed allogeneic bone marrow chimeras. Immunobiology 1995; 194: 376.
36. Kawamura M, Hisha H, Li Y, Fukuhara S, Ikehara S. Distinct qualitative differences between normal and abnormal hemopoietic stem cells in vivo and in vitro. Stem Cells 1997; 15: 56.
37. Sprent J, Hurd M, Schaefer M, Heath W. Split tolerance in spleen chimeras. J Immunol 1995; 154: 1198.

Received 14 February 2001.
Revision Requested 29 June 2001.
Accepted 6 August 2001.

reference 3

Graft-versus-host–reactive donor CD4 cells can induce T cell–mediated rejection of the donor marrow in mixed allogeneic chimeras prepared with nonmyeloablative conditioning

Yong-Mi Kim, Markus Y. Mapara, Julian D. Down, Kevin W. Johnson, Florence Boisgerault, Yoshinobu Akiyama, Gilles Benichou, Michele Pelot, Guiling Zhao, and Megan Sykes

Murine mixed hematopoietic chimerism can be achieved following nonmyeloablative conditioning with cyclophosphamide, T cell–depleting monoclonal antibodies, and thymic irradiation. Donor lymphocyte infusions (DLIs) 35 days after bone marrow transplantation (BMT) convert mixed to full donor chimerism and mediate graft-versus-lymphoma effects without graft-versus-host disease. We evaluated the role of T-cell subsets in DLIs in converting mixed to full donor chimerism in a fully major histocompatibility complex–mismatched strain combination. Whereas DLIs administered on day 35 converted 100% of mixed chimeras to full donor chimerism, conversion was less frequent when either CD4 or CD8 cells were depleted, indicating that both subsets contribute to the conversion. Surprisingly, administration of CD8-depleted DLIs led to complete loss of donor chimerism in a high proportion (54%) of recipients compared with CD4-plus CD8-depleted DLIs (15%) or CD4-depleted DLIs (0%) ($P < .05$). DLIs administered at early time points after BMT (eg, day 21) also precipitated rejection of donor marrow by recipient αβ T cells, in association with donor CD4 cell expansion and high production of interleukin 2 (IL-2), IL-4, and interferon-γ. Thus, DLIs can paradoxically induce marrow rejection by residual host αβ T cells. These results have implications for the timing of and use of subset depletion of DLIs in recipients of nonmyeloablative transplants. (Blood. 2004;103:732-739)

Introduction

In efforts to overcome the toxicities associated with conventional hematopoietic cell transplantation (HCT), nonmyeloablative preparative regimens have been used in various rodent models[1] and in clinical trials.[2-5] We have used mixed chimerism as immunotherapy for hematologic malignancies in a 2-step process involving establishment of donor-specific tolerance by inducing mixed chimerism with nonmyeloablative conditioning that includes monoclonal antibodies (mAbs), which deplete donor and host T cells in vivo. Mixed chimerism then serves as a platform for adoptive immunotherapy using donor lymphocyte infusions (DLIs), which convert mixed to full donor chimeras, and mediate graft-versus-tumor (GVT) effects without causing graft-versus-host disease (GVHD).[6-8] Thus, a lymphohematopoietic graft-versus-host reaction (LGVHR) is used to achieve GVT effects without GVHD.[6-8]

We have now sought to delineate the T-cell subsets that contribute to the LGVHR mediated by DLI in mixed chimeras produced in the fully major histocompatibility complex (MHC)–mismatched B10.A ($H2^a$) → C57BL/B6 ($H2^b$) strain combination using a cyclophosphamide (Cytoxan; CTX)–based nonmyeloablative conditioning regimen,[6] in which durable mixed chimerism and donor-specific immune tolerance is normally achieved.[7] DLI administered on day 35 converts these mixed chimeras to full donor chimeras without causing GVHD.[6] With this regimen, anti-CD4 and anti-CD8 mAbs in the conditioning regimen circulate at high levels for several weeks following bone marrow transplantation (BMT), resulting in in vivo depletion of T cells in the marrow inoculum.[9,10] We have now analyzed the T-cell subsets involved in this conversion by DLIs administered on day 35 and examined the effect of earlier DLI administration on chimerism. The results paradoxically uncover a residual rejection capacity in the host given early DLI or CD8 cell–depleted DLI. Collectively, our results suggest a model wherein graft-versus-host (GVH)–reactive donor T cells may activate residual host T cells and thereby trigger rejection of donor marrow grafts.

Materials and methods

Animals

Female or male B10.A ($H2^a$: K^k, I^k, D^d) donor mice and recipient C57BL/6 ($H\text{-}2^b$: K^b, I^b, D^b), T-cell receptor β (TCRβ) knockout C57BL/6 and B10.RIII ($H\text{-}2^r$: $K^rI^rD^r$) mice were purchased from Frederick Cancer

From the Transplantation Biology Research Center, Bone Marrow Transplantation Section, Massachusetts General Hospital/Harvard Medical School, Boston, MA; Biotransplant, Inc, Medford, MA; and Schepens Eye Institute, Harvard Medical School, Boston, MA.

Submitted March 5, 2003; accepted September 15, 2003. Prepublished online as *Blood* First Edition Paper, September 25, 2003; DOI 10.1182/blood-2003-02-0643.

Supported by National Institutes of Health grants RO1 CA79989, RO1 HL49915, RO1 EY13310, and RO1 AI33704; in part by Mildred-Scheel Stiftung of the Deutsche Krebshilfe (Y.-M.K.); by a grant from Deutsche Forschungsgemeinschaft (DFG Ma-1664/2-1; M.Y.M.); and by a fellowship from the Foundation pour la Recherche Medicale (F.B.). Y.-M.K. and M.Y.M. contributed equally to this work.

Reprints: Megan Sykes, Transplantation Biology Research Center, Bone Marrow Transplantation Section, Massachusetts General Hospital/Harvard Medical School, MGH-East Bldg 149-5102, 13th St, Boston, MA 02129; e-mail: megan.sykes@tbrc.mgh.harvard.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Research Facility, National Cancer Institute (Frederick, MD) or from the Jackson Laboratory (Bar Harbor, ME). Mice were used for in vivo experiments at 8 to 12 weeks of age. All mice were housed in autoclaved microisolator environments, and all manipulations were performed in a laminar flow hood.

Preparation of bone marrow cells and spleen cells

Bone marrow was harvested and single-cell suspensions were prepared as described previously.[11] Donor spleens were harvested and gently teased in ammonium chloride potassium (ACK) lysing buffer (BioWhittaker, Walkersville, MD). Single-cell suspensions were filtered through nylon mesh. Alternatively, single-cell splenocyte suspensions were separated over a Lympholyte-M density gradient (Cedarlane Laboratories, Hornby, ON, Canada).

Transplantation of allogeneic BMCs and SPCs in recipients of nonmyeloablative conditioning

Mixed chimerism was induced in female C57BL/6 ($H2^b$) mice by a nonmyeloablative cyclophosphamide (Cytoxan [CTX])–based regimen as described previously.[6] The regimen includes T cell–depleting anti-CD4 mAb GK1.5 (2.0 mg) and anti-CD8 mAb 2.43 (1.4 mg) intraperitoneally on day −5, CTX (Mead Johnson, Princeton, NJ) 200 mg/kg intraperitoneally on day −1, and 7 Gy thymic irradiation (^{60}Co source or Siemens x-ray machine, operated at 250 kVp, 1.47 Gy/min) on day 0. Twenty million B10.A bone marrow cells (BMCs) were injected intravenously on day 0 as described.[6] DLI consisted of 30×10^6 (final injected number) donor-type (B10.A) spleen cells (SPCs) that were either untreated, complement treated, or depleted of T cells with anti-CD4 mAb GK1.5 or anti-CD8 mAb 2.43 plus low-toxicity rabbit complement, as described previously.[12] T-cell depletion was analyzed by flow cytometry (FCM) and less than 1% residual cells of the depleted subset remained.

In further experiments, male C57BL/6 mice received a modified nonmyeloablative conditioning regimen that included CTX 200 mg/kg given intraperitoneally on day −1 and 7 Gy thymic irradiation on day 0 prior to transplantation of 15×10^6 male B10.A BMCs and a total of 10×10^6 T-cell subset–depleted (with GK1.5 or 2.43 plus sheep antirat IgG magnetic beads [Dynal, Lake Success, NY]) or undepleted B10.A SPCs given intravenously on day 0. The recipient mice were treated with either anti-CD4 mAb GK1.5 (2.0 mg) or anti-CD8 mAb 2.43 (1.4 mg) or both mAbs on day −5, or received no T cell–depleting mAbs.

Assessment of chimerism

Chimerism in white blood cells (WBCs) was assessed as described[9,10] by 2-color FCM using a Becton Dickinson FACScan cytometer (Mountain View, CA) and analyzed to determine the levels of donor-type hematopoietic reconstitution. WBCs were prepared and analyzed for lineage chimerism by FCM after staining with biotinylated anti-Dd mAb 34-2-12 (prepared in our laboratory) plus phycoerythrin (PE)–conjugated streptavidin (PEA) or Cychrome streptavidin (CycSA) versus anti–CD4-fluorescein isothiocyanate (FITC), anti–CD8β-FITC, anti–B220-FITC (all purchased from PharMingen, San Diego CA), anti–Mac-1-FITC (Caltag, San Francisco, CA), anti–CD3ε-PE, anti–CD4-PE, anti–CD8-PE, anti–B220-PE, or anti–CD11b(Mac-1)–PE (PharMingen). Nonreactive control mAb HOPC1-FITC (mouse IgG2a prepared in our laboratory) and rat IgG2a-PE (Pharmingen) were used as negative controls. To reduce nonspecific binding, 10 μL mAb 2.4G2 (anti–Fcγ-RII/III receptor, CDw32) hybridoma supernatant was added.[13] The relative percentage of donor cells in a chimera was calculated using the formula: 100% × (donor phenotype percentage positive-isotype control)/[(donor phenotype positive-isotype control) + (recipient phenotype percent positive-isotype control)]. Propidium iodide (PI) staining and live gating on PI^- cells were performed. Ten thousand events were collected and analyzed.

Mixed lymphocyte reactions and cell-mediated lympholysis assays

These were performed by standard techniques as we have previously described.[7]

ELISA spot assays

The 96-well enzyme-linked immunosorbent assay (ELISA) spot plates (Polyfiltronics, Rockland, MA) were coated with a capture mAb in sterile phosphate-buffered saline (PBS) overnight. Anti–interleukin 2 (IL-2), anti–interferon γ (IFN-γ), and anti–IL-4 capture mAbs were used at 3, 4, and 2 μg/mL, respectively (PharMingen). On the day of the experiment, the plates were washed twice with sterile PBS, blocked for 1.5 hours with PBS containing 1% bovine serum albumin (BSA), then washed 3 times with sterile PBS. Responder cells were added to wells previously filled with stimulator cells as previously described.[14] Cells were incubated for different periods of time, depending on the cytokine measurement: 20 hours for IL-2 and 42 hours for IFN-γ and IL-4. The plates were washed 3 times with PBS, then 4 times with PBS containing 0.025% Tween (PBST). Biotinylated antilymphokine detection mAbs were added at 2 μg/mL (PharMingen) and incubated either for 5 hours at room temperature or overnight at 4°C. After washing 3 times with PBST, avidin-horseradish peroxidase (1:2000) was added to each well for 1.5 hours. Four washes with PBS were performed before the spots were revealed by the addition of the developing solution composed of 800 μL 3-amino 9-ethylcarbazole (AEC; Sigma-Aldrich, St Louis, MO; 10 mg dissolved in 1 mL dimethylformamide) in 24 mL 0.1 M sodium acetate, pH 5.0, catalyzed by 12 μL H_2O_2. The resulting spots were counted and analyzed on a computer-assisted ELISA spot image analyzer (CTL, Cleveland, OH).

Statistical analysis

Survival data were analyzed using the log-rank test. Differences between group weights were tested using Mann-Whitney U test or Student t test of means. The Fisher test was used to compare the incidence of rejection and conversion to full chimerism. A paired t test was used to compare antigen-specific cytokine responses to background in the ELISPOT assay.

Results

Both CD4 and CD8 T cells in DLIs contribute to conversion of mixed to full donor chimerism, but CD8 cells play a predominant role

To evaluate the role of T-cell subsets in DLIs administered on day 35 in the conversion from mixed to full donor chimerism, we established mixed chimerism in C57BL/6 mice ($H2^b$) that were conditioned as described[6] with our nonmyeloablative regimen followed by administration of 20×10^6 B10.A ($H2^a$) BMCs on day 0. Similar to previous results, the majority (63 of 64, 98.4%) of these animals achieved low levels of mixed hematopoietic chimerism. On day 35, DLIs consisting of 30×10^6 SPCs treated with complement only (C′ control) or depleted in vitro of the CD4 or CD8 T-cell subset were administered. Donor CD4, CD8, B-cell, monocyte, and granulocyte chimerism was assessed in peripheral WBCs until 16 or 37 weeks after BMT, when the animals were killed. Because the granulocyte and monocyte lineages showed similar chimerism levels, as did CD4, CD8, and B cells, donor granulocyte chimerism is depicted in Figure 1 to represent myeloid chimerism and CD4 T-cell data are presented to represent lymphoid lineages. Pooled data are presented from 2 different experiments, each of which produced similar results.

WBCs of peripheral blood in the control group receiving no DLIs showed generally stable but low levels of donor chimerism in all lineages (Figures 1A-B and 2A). Complement-treated control DLIs led to conversion to full (100%) donor chimerism in all animals by 11 weeks after BMT (Figures 1C-D and 2A). CD4 cell–depleted ($CD4^-$) DLIs led to a marked increase in donor myeloid and lymphoid chimerism in 7 of 12 animals, and 5 converted to full donor chimerism (Figures 1E-F and 2A). Among

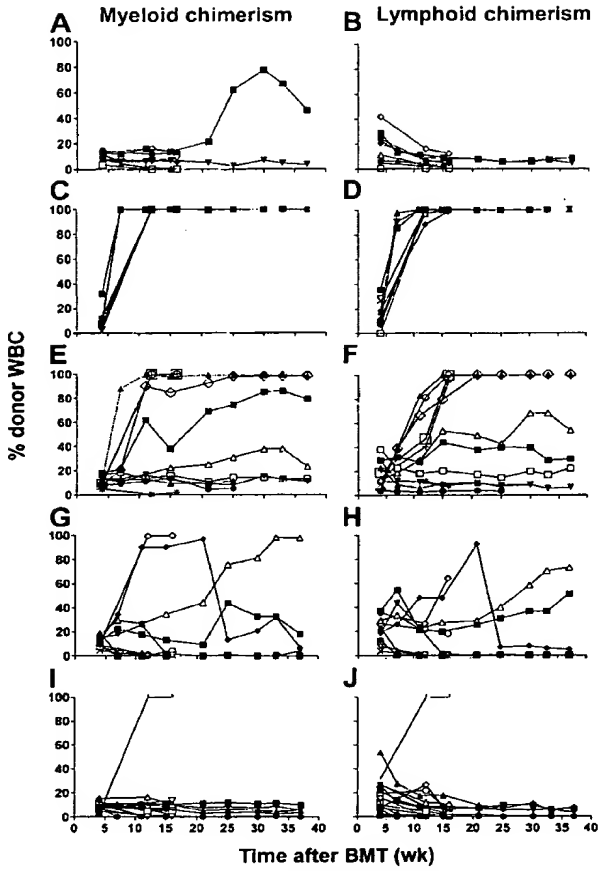


Figure 1. Both CD4 and CD8 T-cell subsets contribute to conversion to full chimerism, whereas CD8 cells play a requisite role. (A-J) Time course of individual donor granulocyte chimerism (representative of both myeloid lineages followed; left panels) and donor $CD4^+$ chimerism (representative of all lymphoid lineages followed; right panels). WBCs chimerism is shown for C57BL/6 (H2b) mice treated with CTX 200 mg/kg on day −1 and 7 Gy thymic irradiation on day 0 that received 20×10^6 BMCs on day 0 and a final number of 30×10^6 T-cell subset–depleted or undepleted SPCs on day 35 after BMT from B10.A ($H2^a$) mice. Recipients were given no DLI (n=9; A-B), complement only–treated DLI (n=11; C-D), CD4 cell–depleted DLI (n=12; E-F), CD8 cell–depleted DLI (n=11; G-H), or CD4 and CD8 cell–depleted DLI (n=20; I-J). Each line represents an individual animal in each panel. Data were pooled from 2 independent experiments.

the recipients of CD8-depleted ($CD8^-$) DLIs (Figures 1G-H and 2A), only 4 of 11 showed fluctuating increases in donor myeloid and lymphoid chimerism, and none converted to full donor chimerism. One (5%) of 20 animals in the group receiving DLIs depleted of CD4 and CD8 subsets ($CD4^-8^-$ DLIs) showed conversion to full myeloid and lymphoid donor chimerism (Figures 1I-J and 2A), but the group otherwise showed stable chimerism, similar to controls receiving no DLI. We killed several animals from each experimental group to assess donor engraftment in various organs (bone marrow, spleen, and lymph nodes). FCM analysis showed similar donor chimerism in various lineages to that observed in peripheral WBCs (data not shown). Consistent with our previous results,[6] none of the animals showed signs of GVHD in this experiment, weight loss, or death (data not shown). Thus, whereas both CD4 and CD8 cells in DLIs contribute to conversion to full chimerism, CD8 cells play a predominant role, and this conversion occurs without observable GVHD when DLIs are given 35 days after BMT.

Administration of donor CD4 cells without CD8 cells in DLIs paradoxically induces loss of donor chimerism

Surprisingly, we observed a complete loss of donor chimerism (to ≤ 0.7% donor WBCs following previously stable chimerism) in a high percentage (54.4%, 6 of 11) of recipients of $CD8^-$ DLIs (Figures 1G-H and 2B). The incidence of loss of chimerism in the group not receiving any DLI was only 22.2% (2 of 9; Figures 1A-B and 2B). In mice receiving $CD4^-8^-$ DLIs, loss of donor chimerism occurred in only 15% (3 of 20) of animals (Figures 1I-J and 2B; $P < .05$ compared with $CD8^-$ DLI group). Loss of chimerism was not seen in recipients of $CD4^-$ DLIs (0 of 12; Figures 1E-F and 2B), or in recipients of C′ control DLIs (0 of 11; Figures 1C-D and 2B; $P < .05$ compared with recipients of $CD8^-$ DLIs). In animals that lost detectable chimerism in WBCs, chimerism was also undetectable in the bone marrow, spleen, and lymph node cells when the animals were killed 37 weeks after BMT (2, 5, or 6 animals/group, data not shown).

The significantly higher incidence of loss of chimerism in recipients of $CD8^-$ DLIs compared with recipients of $CD4^-8^-$ DLIs ($P < .05$) demonstrates that donor CD4 cells administered without CD8 cells in DLIs induce loss of donor chimerism.

Unseparated DLIs administered on day 21 induce loss of donor chimerism

We compared the effect of DLIs administered at various times in groups of stable chimeras in the B10.A → B6 strain combination. As in previous experiments, animals not receiving DLIs remained chimeric for the duration of follow-up (Figure 3A left panels). DLIs administered on day 35 led to conversion to full donor chimerism (Figure 3A right panels). Strikingly, DLIs administered on day 21 led instead to a complete loss of chimerism in 5 of 6 recipients (Figure 3A middle panels). Similar results were seen with day 21 DLIs in 5 of 5 experiments. A total of 23 (72%) of 32 mice lost measurable chimerism completely after these DLIs compared with only 5 (15%) of 33 non-DLI recipients ($P < .0005$). Animals that lost chimerism following day-21 DLIs also failed to demonstrate long-term tolerance in mixed lymphocyte reactions (MLRs) and cell-mediated lympholysis (CML) assays performed as described[7] at the time the animals were killed, whereas control chimeras showed specific tolerance in these assays (data not shown).

To determine whether or not host T cells were responsible for the paradoxical loss of chimerism that occurred when DLIs were given on day 21, we compared the effect of these DLIs in mixed chimeras prepared in wild-type B6 mice to that in chimeras prepared in TCRβ-deficient (TCRβ knockout, KO) recipients, which completely lack $\alpha\beta$ TCR^+ T cells.[15] As is shown in Figure 3B, multilineage mixed chimerism developed to similar levels prior to DLI in wild-type and TCRβ KO B6 mice. DLI again led to a complete loss of chimerism in wild-type B6 mice. In contrast, TCRβ KO mice showed an increase in chimerism after 5 weeks, and this pattern was not influenced by the administration of DLIs on day 21 (not significant [NS] at all time points except B-cell chimerism at 5 weeks, $P = .04$). All TCR KO mice remained chimeric in marrow and spleen when killed 22 or 30 weeks after BMT. No difference in chimerism was seen between TCR KO chimeras that did or did not receive DLIs (eg, mean ± SD, 14.4% ± 4.6% B-cell chimerism in non-DLI versus 17.0% ± 13.0% in DLI group). Among wild-type chimeras, only non-DLI recipients showed any chimerism at death (5 of 8 chimeric; mean ± SD, 6.3% ± 5.0% B-cell chimerism), and the level was significantly lower than that in TCR KO chimeras ($P < .05$). Thus, we conclude

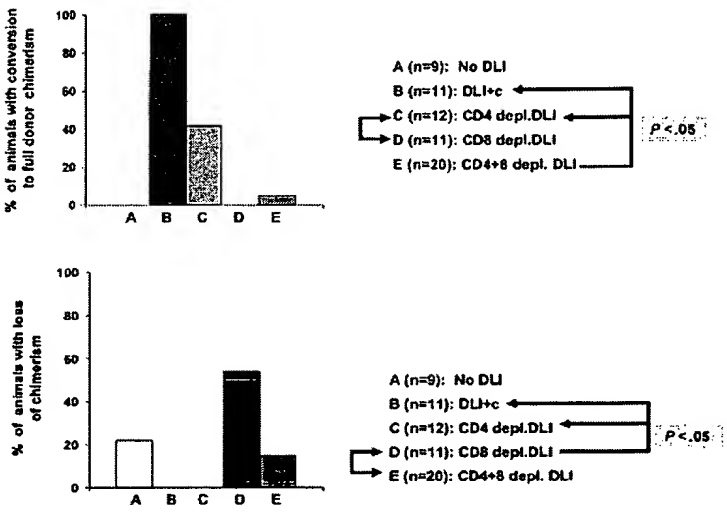


Figure 2. CD4 cells given with CD8 cells in day-35 DLIs promote conversion to full donor chimerism, but CD4 cells given without CD8 cells promote loss of chimerism. The percentage is shown of animals with conversion to full donor chimerism (A) or with loss of detectable donor chimerism (B) following various DLIs. The same animals presented in Figure 1 are presented here. Recipients were given no DLI (n=9; group A, No DLI), complement only–treated DLI (n=11; group B, DLI + c), CD4 cell–depleted DLI (n=12; group C, CD4 depl. DLI), CD8 cell–depleted DLI (n=11; group D, CD8 depl. DLI), or CD4 and CD8 cell–depleted DLI (n=20; group E, CD4 + 8 depl. DLI). Each bar represents one group in each panel. Data were pooled from 2 independent experiments. The difference in percentages of animals with conversion to full chimerism was statistically significant for group E versus group B or group C; for group C versus group D and for group B versus group D. The difference in percentages of animals with loss of chimerism was statistically significant for group D versus group B, group C, and group E. In the 2 separate experiments, the following numbers of mice converted to full donor chimerism in experiments 1 and 2, respectively: group A, 0 of 6, 0 of 3; group B, 8 of 8, 3 of 3; group C, 3 of 4, 2 of 8; group D, 0 of 4, 0 of 7; group E, 1 of 15, 0 of 5. In the 2 separate experiments, the following numbers of mice lost chimerism in experiments 1 and 2, respectively: group A, 1 of 6, 1 of 3; group B, 0 of 8, 0 of 3; group C, 0 of 4, 0 of 8; group D, 2 of 4, 4 of 7; group E, 2 of 15, 1 of 5.

that recipient αβ T cells are responsible for the loss of chimerism that occurs following day-21 DLIs in wild-type B6 recipients.

Alloresponses in the first 5 weeks after BMT in control chimeras and day-21 DLI recipients

The above results suggested that T cells capable of rejecting the donor were present in mixed chimeras 21 to 35 days after BMT. We therefore evaluated alloreactivity by preparing mixed chimeras and killing them on day 21, 28, or 35 to evaluate CML, MLR, and ELISPOT responses to donor, host, and third-party antigens, and to determine the effects of day-21 DLIs on these responses.

On day 21, antidonor, antihost, and anti–third-party MLR responses of splenocytes in 2 of 5 mice not receiving DLIs were similar to those of naive B6 (host-type) mice, whereas 3 chimeras were globally unresponsive. By day 28, chimeras that did not receive DLIs showed unresponsiveness to donor and host, with absent or poor responses to third-party antigens. Even by day 35, poor responses to third-party antigens were seen (data not shown).

Day 21 DLI was associated with the presence of measurable antihost MLR responses in 3 of 5 animals, antidonor responses in 1 of 5, and anti–third-party responses in 4 of 5 mice on day 28. By day 35, 1 of 4 DLI recipients responded to all 3 stimulators, and the remaining 3 were generally unresponsive (data not shown).

We also performed CML assays on the day-35 splenocyte populations. The non-DLI chimeras were all unresponsive to

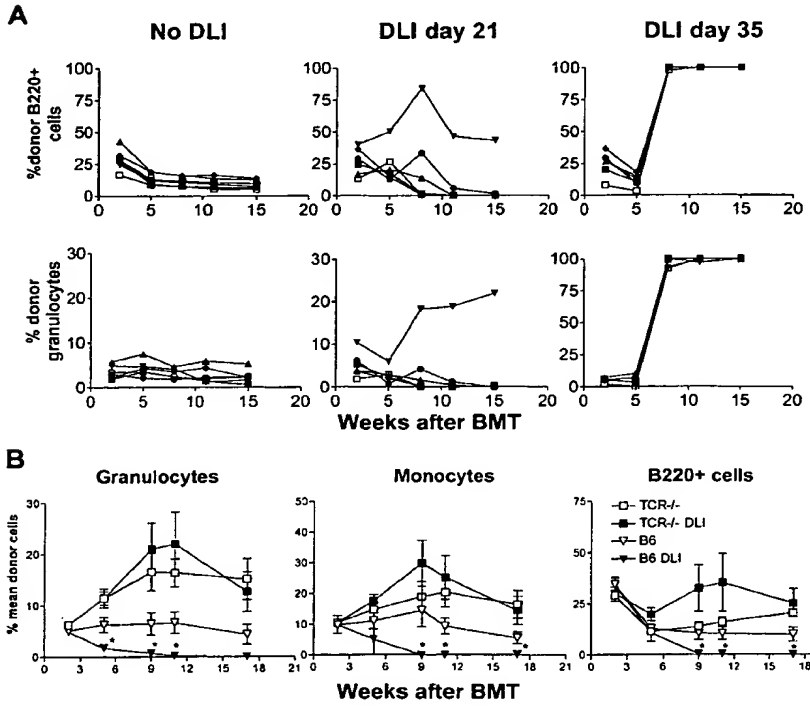


Figure 3. Effect of timing of DLI on changes in chimerism. (A) Loss of chimerism induced by day-21 DLI. Results of one of 5 similar experiments are shown. Each line represents an individual animal; 6 animals/group. (B) Requirement for host αβ T cells for loss of chimerism induced by day-21 DLI. Means ± SD are shown. Wild-type recipients (B6): No DLI, n=6; DLI (30×10^6 SPCs), n=8. TCRβ KO ($TCR^{-/-}$) recipients: No DLI, n=7; DLI (30×10^6 SPCs), n=7. *Denotes statistically significant difference between DLI and no DLI groups.

Table 1. Splenic chimerism and lymphocyte subsets

	B cells, mean ± SD		CD4 cells, mean ± SD		CD8 cells, mean ± SD	
	Donor	Host	Donor	Host	Donor	Host
No DLI, n = 3	10.89 ± 1.28	61.13 ± 7.63	1.05 ± 0.15	9.58 ± 3.72	0.01 ± 0.01	0.72 ± 1.09
DLI, n = 5	10.28 ± 2.94	53.68 ± 5.32	4.18 ± 0.94	6.24 ± 1.93	0.02 ± 0.01	0.05 ± 0.04
P	NS	NS	< .005	NS	NS	NS

donor, host, and third-party antigens (data not shown), consistent with the poor recovery of CD8 T cells seen at 4 weeks (Table 1). Whereas 3 of 4 DLI recipients produced similar results, one DLI recipient showed a weak, but measurable antidonor CML response (percent specific lysis 17.7% at 100:1 responder-target ratio), despite the absence of an anti–third-party CML response (data not shown).

The above data did not strongly implicate a cytotoxic T-lymphocyte (CTL)–mediated effector mechanism of rejection of donor marrow in recipients of day-21 DLIs, suggesting that cytokines might instead be responsible. Results of ELISPOT assays performed 7 days following DLI (day 28) were consistent with this possibility.

As shown in Figure 4, day-28 spleens of control (non-DLI) mixed chimeras (n = 6) did not respond above baseline (background) to B6 (host-type) stimulators. However, a weak (compared with that of naive B6 mice) but significant IL-2 response to donor antigens was detected ($P < .05$). Strong anti–third-party IL-2 responses above baseline were detected ($P < .001$), and these were similar to the responses of naive B6 and B10.A mice. IL-4 responses of chimeras to third-party antigens were significantly greater than baseline ($P < .01$ by paired *t* test) and were also similar to those of naive B6 and B10.A controls, whereas the chimeras were specifically unresponsive to donor and host antigens. A weak but significant IFN-γ response to third-party stimulators ($P < .05$ versus baseline) was detected among chimeras, which demonstrated complete unresponsiveness to donor and host antigens. In summary, the non-DLI chimeras demonstrated specific cytokine hyporesponsiveness (with a weak antidonor IL-2 response) or complete, specific unresponsiveness (IFN-γ, IL-4) toward the donor and host by day 28.

Strikingly, the chimeric animals receiving DLIs on day 21 (n = 9) showed significantly increased baseline IL-2–producing ($P < .01$) and IFN-γ–producing ($P < .005$) cells compared with non-DLI controls. No significant difference was detected between the groups in baseline IL-4–producing cell numbers. Although average IL-2–producing cell numbers did not increase significantly in response to donor or host antigens, paired analysis revealed significant responses to donor, host, and third-party antigens compared with the baseline ($P < .05$, $< .005$, $< .0001$, respectively) in the absence of in vitro stimulation. A similar situation prevailed for IL-4–producing cells ($P < .005$, $< .01$, $< .0001$ by paired *t* test for responses to donor, host, and third-party antigens), but not for IFN-γ, in which significant ($P < .05$) responses to third-party but not donor or host antigens were detectable by paired comparison to baseline values. In summary, day-21 DLIs led to a marked increase in IL-2– and IFN-γ–producing cells, with clear evidence for loss of tolerance to host and donor antigens for IL-2– and IL-4–producing cells.

An analysis of splenic chimerism in the groups analyzed on day 28 revealed an absence of donor CD8 cells and very few host CD8 cells, and the presence of relatively high percentages of donor and recipient CD4 cells (Table 1). DLIs on day 21 did not lead to a change in B-cell or CD8 T-cell chimerism, but did lead to a significant ($P < .005$) increase in the percentage of donor CD4 cells in the spleen on day 28. Absolute numbers of donor CD4 cells ($P < .01$) but not of recipient CD4 cells or of donor or recipient CD8 cells were increased in DLI recipients compared with controls. These results are consistent with the possibility that donor CD8 cells in day-21 DLIs were depleted by residual anti-CD8 mAb in the serum, whereas DLI CD4 cells were not depleted and were able to proliferate as a result of the GVH alloresponse. By FCM

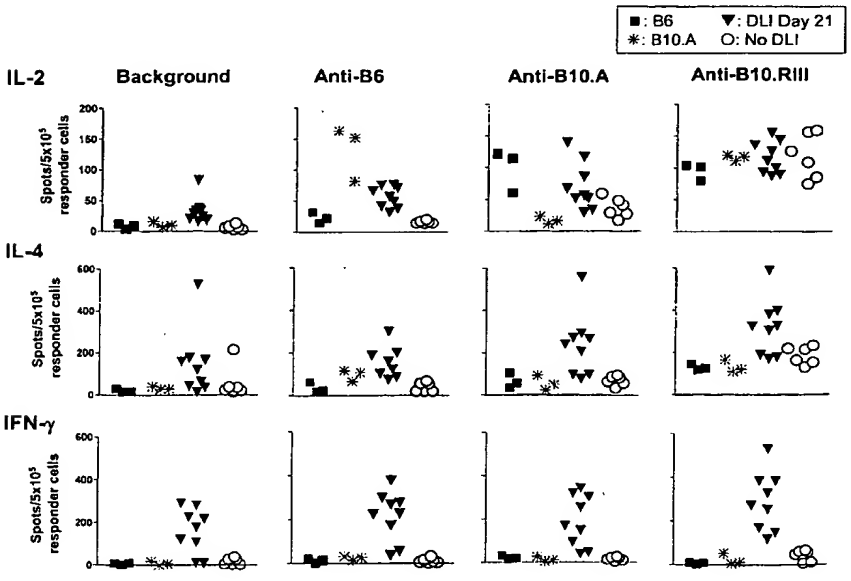


Figure 4. ELISPOT analysis of cytokine production in response to donor (B10.A), host (B6), and third-party (B10.RIII) alloantigens by day 28 splenocytes of mixed chimeras receiving no DLI or DLIs on day 21 after BMT. Results of 2 similar experiments are shown. Each symbol represents an individual animal. The upper panel shows IL-2–producing cells, the middle panel shows IL-4–producing cells, and the lower panel shows IFN-γ–producing cells among SPCs from control naive B6 mice (n=3; ■), naive B10.A mice (n=3; *), chimeras receiving DLI on day 21 (DLI day 21; n=9; ▼) or not receiving DLI (no DLI; n=6; O). Chimeric SPCs were collected on day 28. The in vitro stimulators are listed from left to right: medium (background in the absence of stimulator cells in vitro), B6, B10.A, and B10.RIII.

analysis, anti-CD4 and anti-CD8 mAbs were both present in the undiluted serum on day 21 at levels that saturated all mAb-binding sites on normal splenocytes (data not shown).

Evidence that donor CD4 cells have potential to inhibit engraftment of marrow given on day 0

We next examined the effect of donor and recipient T-cell subsets present at the time of BMT on engraftment and GVHD. For this purpose the conditioning regimen was modified so that mice received either no T-cell–depleting mAbs, or only anti-CD4, anti-CD8, or both mAbs on day −5, and donor SPCs were administered on day 0. B6 mice received CTX 200 mg/kg on day −1, 7 Gy thymic irradiation on day 0, and 15×10^6 BMCs and 10×10^6 SPCs from B10.A donors on day 0.

Control recipients (n = 5) of donor SPCs on day 0 without T cell–depleting mAbs showed no donor engraftment at any time (data not shown) and remained healthy with continuous weight gain (not shown). In contrast, among recipients of CD4-depleting mAb in vivo (n = 6), nearly complete or complete donor myeloid chimerism (95%-99%) was achieved by week 3 after BMT in 5 of 6 mice (Figure 5A-B). Full chimerism was sustained long-term in 3 of the 5 mice. One animal died early of an anesthetic overdose and 2 animals died, at 12 and 20 weeks after BMT, with 20% to 30% body weight loss and clinical signs of GVHD (ruffled fur, skin lesions). Among the 3 remaining animals, the 2 with nearly complete donor chimerism also had weight loss and clinical evidence of GVHD, whereas the nonchimeric animal remained healthy and gained weight (data not shown).

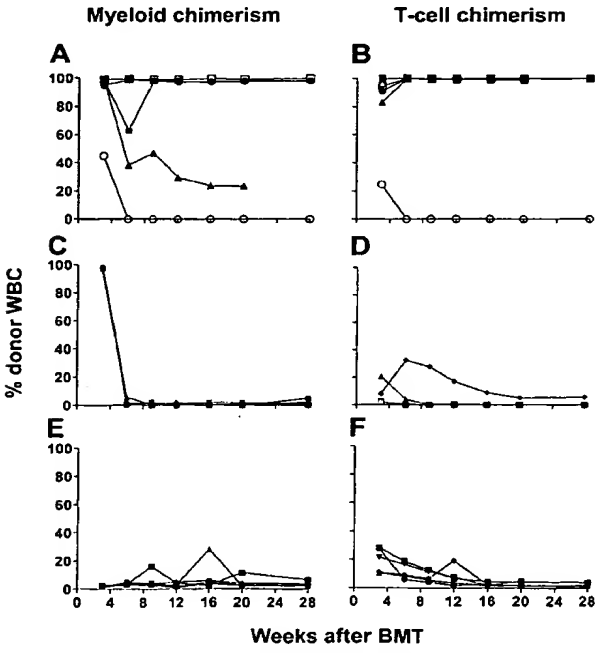


Figure 5. In vivo CD4 depletion of BMCs and DLIs administered on day 0 facilitate full donor myeloid and lymphoid engraftment. (A-F) Time course of individual MAC-1$^+$ donor myeloid cell (left panels) and CD3$^-$ donor T cell (right panels) chimerism in WBCs of B6 ($H2^b$) mice treated with CTX 200 mg/kg on day −1 and 7 Gy thymic irradiation on day 0 followed by 15×10^6 BMCs and 10×10^6 SPCs on day 0 from B10.A ($H2^a$) donor mice. Five days before BMT, recipients were given anti-CD4 mAb alone (n=6; A-B), anti-CD8 mAb alone (n=6; C-D), or anti-CD4 and anti-CD8 mAbs (n=5; E-F). Each line represents an individual animal in each panel. One of 2 experiments is presented.

In contrast to these results, in vivo depletion of CD8$^+$ cells (n = 6) was associated with sustained, low-level donor engraftment in only 1 of 6 mice (Figure 5C-D). One animal with 38.4% weight loss prior to death at 4 weeks after BMT was not assessed for chimerism. All other animals survived without weight loss and remained clinically healthy (data not shown).

All recipients of combined CD4- and CD8-depleting mAbs in vivo (n = 5) engrafted with low donor myeloid (2%-6.7%; Figure 5E) and lymphoid chimerism (1.6%-3.9%; Figure 5F), which persisted until at least week 28 after BMT. Thus, sustained donor engraftment occurred most frequently in association with in vivo depletion of CD4 plus CD8 cells, but a high incidence was also seen in recipients depleted only of CD4 cells. Engrafted animals in the latter group showed high levels of donor chimerism, and this condition was associated with clinical evidence of GVHD. In contrast, the combined depletion of CD4 and CD8 T cells permitted mixed chimerism to be achieved in the absence of clinically apparent GVHD.

In a repeat experiment, 5 of 8 recipients treated with no mAb showed no chimerism, whereas the remaining 3 showed low levels (< 15%) of myeloid and lymphoid mixed chimerism at 3 weeks, which disappeared by 6 weeks. In contrast, most animals (6 of 7) treated with anti-CD4 mAb showed low (< 20% donor) myeloid and lymphoid chimerism at 3 weeks ($P < .05$ versus no mAb group for B cells, monocytes, and granulocytes), which persisted at 6 weeks and disappeared by week 9. These mice remained healthy. Only 1 of 8 recipients of anti-CD8 mAb showed initial low (≤ 5%) donor myeloid chimerism (NS versus no mAb group for any lineage), which was lost by 6 weeks. Eight of 8 mice treated with anti-CD4 plus anti-CD8 showed a low level (eg, mean 6% donor B cells at 6 weeks) of chimerism. All animals showed weight gain, with no clinical evidence of GVHD (data not shown). Thus, although no full chimerism or GVHD was seen in this experiment, the observation that in vivo CD4 depletion increased donor engraftment compared with no mAb treatment or anti-CD8 mAb treatment was reproduced.

The results described, in which CD4 depletion in vivo was required to permit sustained engraftment of donor marrow in most animals, suggested that recipient CD4 cells might be capable of rejecting donor marrow, even in the absence of CD8 cells. However, given that the in vivo depletion affected both donor and host CD4 cells, our results did not rule out the possibility that donor CD4 cells might paradoxically inhibit donor engraftment, in a manner analogous to the effects described with day-35 DLIs. We therefore evaluated engraftment in mice not receiving in vivo T-cell depletion, but receiving B10.A BMCs and a total of 10×10^6 donor SPCs that were or were not depleted of CD4 or CD8 T cells following treatment with CTX 200 mg/kg on day −1 and 7 Gy thymic irradiation on day 0.

The control animals receiving CTX, thymic irradiation, BMCs, and untreated SPCs (n = 5) on day 0 again showed no donor engraftment. Five of 5 animals receiving CD4 cell–depleted SPCs on day 0 developed chimerism, at high (> 95% donor) levels in 3 mice and at lower levels in the other 2. Clinical GVHD was not apparent. None of 5 recipients of CD8-depleted SPCs showed any chimerism, nor did any recipients of CD4-depleted plus CD8-depleted SPCs on day 0. In a repeat experiment involving 8 animals per group, however, none of the mice in any group developed measurable chimerism at any time. Thus, although there was variability from experiment to experiment, these results suggest that donor CD8 cells given on day 0 are able to promote donor engraftment in recipients not depleted of either CD4 or CD8 T

cells. However, the presence of donor CD4 cells in these inocula may interfere with this engraftment-promoting ability of donor CD8 cells.

Discussion

We have previously demonstrated that GVH alloresponses induced by DLI can be confined to the lymphohematopoietic system, where they can convert mixed to full donor chimerism and mediate GVT effects without causing GVHD.[6,8,16,17] We have now performed studies to identify the T-cell subset(s) responsible for this conversion in the setting of a full MHC mismatch in mice undergoing nonmyeloablative BMT. Our data demonstrate that both CD4 and CD8 cells contribute to conversion to full chimerism following day-35 DLI. CD8 cells play a requisite role in this conversion and can mediate conversion even when CD4 cells are depleted. Nevertheless, CD4 T cells significantly increased the frequency with which conversion to full chimerism occurred following DLI, indicating a contribution of CD4 cells to this process.

In addition, these studies have revealed a paradoxical effect of CD4 cells in DLIs. When CD4 cells were given in day-35 DLIs without CD8 cells, disappearance of measurable chimerism was induced in the majority of animals. This effect was dependent on the presence of CD4 T cells in the DLIs because chimerism was not affected by the administration of CD4-depleted plus CD8 cell–depleted DLI. This result demonstrates that provision of donor antigen-presenting cells (APCs) in DLIs is not responsible for inducing loss of chimerism, because the incidence of long-term stable chimerism was similar in recipients of no DLIs and those receiving CD4-depleted plus CD8 cell–depleted DLIs.

Studies involving the administration of donor lymphocytes on day 0, although somewhat variable from experiment to experiment, are consistent with the interpretation that donor CD4 cells can impair engraftment in the presence of residual host immunity. In 2 of 2 experiments, administration of anti-CD4 mAb in vivo without anti-CD8 mAb increased the level of engraftment over that achieved in controls not receiving mAb and that in recipients of anti-CD8 mAb alone. Thus, donor CD8 T cells have the potential to overcome the resistance imposed by recipient T cells, which were largely $CD8^+$ in mice receiving CD4 cell–depleting mAb. Results of studies involving administration of ex vivo T-cell subset–depleted allogeneic SPCs on day 0 to mice that did not receive any mAbs in vivo, though also variable, are consistent with the interpretation that donor CD8 cells have the potential to overcome host resistance mediated by both $CD4^+$ and $CD8^+$ recipient T cells, and that donor CD4 cells interfere with this engraftment-promoting ability of donor CD8 cells. The variable outcomes (engraftment and GVHD) within and between experiments involving donor SPC administration on day 0 to mice not receiving both anti-CD4 and anti-CD8 mAbs in their conditioning suggests that the balance between host-versus-graft (HVG) and GVH responses is very fine in this setting and can be tipped in one or the other direction by unknown variables. In contrast, engraftment is much more reliably achieved, and without GVHD, in mice receiving both CD4-depleting and CD8-depleting mAbs as part of their conditioning.

One possible mechanism for the deleterious effect of donor CD4 cells on donor graft survival under the above conditions is that the GVH reaction induced by CD4 cells provides "help" that activates residual nontolerant host lymphocytes to reject donor marrow. Evidence for the presence of such recipient cells was obtained in ELISPOT assays in non-DLI recipients, in which a significant, though weak, IL-2 response to the donor was detectable on day 28. DLIs given at 21 days after BMT induced loss of chimerism, and this rejection, which was shown to require $\alpha\beta$ T cells, was associated with increased IFN-γ– and IL-2–producing effector cells in freshly isolated splenocyte populations. Additionally, IL-2 and IL-4 assays demonstrated a loss of hyporesponsiveness or tolerance to both donor and host in DLI recipients, consistent with the existence of an activated, bidirectional alloresponse in these mice. Some animals also showed antidonor and antihost MLR responses. Although anti-CD8 and anti-CD4 mAbs were still circulating at high levels at this early time point of DLI (day 21), our previous studies have shown more rapid clearance of anti-CD4 than anti-CD8 mAbs in animals receiving this mAb regimen.[9] The observation that donor CD4 cells but not CD8 cells were expanded on day 28 in spleens of mice that received day-21 DLI (Table 1) suggests that CD8 cells in the DLI were depleted by the residual circulating mAbs, whereas DLI CD4 cells survived and proliferated in the GVH reaction. The MLR responses and cytokine production detected in response to recipient (in addition to donor) antigens in these mice is consistent with the response of a GVH-reactive Th cell population in the DLI, which may have helped to trigger the HVG-reactive Th population that produced cytokines in response to the donor. Evidence does not suggest a cytolytic mechanism of donor marrow rejection following early DLIs, because CTL responses to the donor were not detectable in most cases on day 35, and very few recipient CD8 T cells were detected in the spleens by day 28. The ELISPOT and MLR data favor a cytokine-mediated mechanism of donor marrow rejection, and this may be a counterpart of the IFN-γ–dependent pathway that has been reported to be capable of destroying host hematopoiesis under other conditions.[18] The pathway by which the CD4-mediated GVH reaction elicits this HVG response remains to be elucidated.

In other experiments (data not shown), we have observed that DLIs administered on day 25 can also cause a complete loss of chimerism in a strain combination (B10.BR → BALB/c) in which day 35 DLI converts the animals to full donor chimerism. Although long-term mixed chimeras prepared with this nonmyeloablative regimen are systemically and specifically tolerant of their donors by an intrathymic deletional mechanism,[7] the initial depletion of recipient T cells with this conditioning regimen may be incomplete. Additional mechanisms, such as anergy and regulation, may evolve over time (> 3.5 weeks) to result in the tolerization of residual host T cells, resulting in an initially precarious state of tolerance. If this state is left unperturbed, tolerance ultimately ensues.

The observation that CD8 cell–depleted DLIs on day 35 can precipitate loss of chimerism in recipients undergoing nonmyeloablative BMT may have clinical relevance, because CD8-cell depletion has been evaluated in efforts to achieve graft-versus-lymphoma (GVL) without GVHD from DLI in recipients of HLA-identical donor transplants.[19,20] Conversion of mixed to full donor chimerism was observed in these studies.[21] However, we have not evaluated the effect of subset-depleted DLIs in mice undergoing myeloablative BMT, in which host resistance to the donor may be more fully eliminated than in our CTX-based nonmyeloablative regimen. Furthermore, we have not evaluated the effect of T-cell subset depletion of DLI in MHC-identical murine combinations because naive DLIs are incapable of converting mixed to full donor chimeras in recipients of MHC-matched, multiple minor antigen-mismatched marrow with this nonmyeloablative conditioning regimen in some strain combinations (Y.-M.K. and M.S., manuscript in preparation; J.D.D. et al, unpublished data, August 2001).

Several groups have previously demonstrated that donor CD8 cells can help to promote marrow engraftment in mice,[11,22-24] and that this effect is due, in large part but not entirely, to the ability of GVH reactive CD8 T cells to destroy host lymphocytes that otherwise resist engraftment.[25] Consistently, patients receiving CD8 cell–depleted bone marrow have shown a higher graft rejection rate than recipients of unmodified marrow.[26] However, in sublethally irradiated mice receiving BM transplants from donors differing only at a class II MHC locus, donor $CD4^+$ T cells can eliminate host hematopoietic cells via an IFN-γ–dependent mechanism,[18] causing death due to hematopoietic failure.[27] Our studies provide the first demonstration that, in the presence of residual host immune resistance to the donor, donor CD4 cells can paradoxically help to trigger the rejection process. Nevertheless, in established mixed chimeras, when CD4 T cells are given along with CD8 cells in DLIs, they help to promote conversion to full donor chimerism.

In recipients of donor SPCs on day 0 with in vivo CD4 depletion, animals with full chimerism all showed clinical evidence of GVHD. This did not occur in animals without engraftment or in sustained chimeras produced via in vivo depletion of both CD4 and CD8 cells, suggesting that CD8 cells given on day 0 may have caused GVHD. No GVHD has ever been observed in recipients of 3-fold larger DLIs on day 35 in this strain combination in these or previous studies.[6] The ability to induce GVHD with CD8 T cells administered on day 0 but not with such cells administered in DLIs on day 35 is further evidence of the importance of host recovery from conditioning in conferring resistance to GVHD by DLI.

Thus, the studies here introduce a new concept in HCT. In recipients in which significant HVG alloreactivity persists, the administration of GVH-reactive donor CD4 cells may induce donor graft rejection. Interactions between GVH- and HVG-reactive T cells must now be considered in evaluating the immunobiology of nonmyeloablative HCT. Further studies are needed to dissect the mechanism of this effect and evaluate it in the setting of various histoincompatibilities, which may have important implications for nonmyeloablative HCT and the use of T-cell subset–depleted DLIs in attempts to achieve GVL effect without GVHD.

Acknowledgments

We thank Ms Robin Laber for expert assistance with the manuscript, Drs Thomas Spitzer and Ronjon Chakraverty for critical reading of the manuscript, and Mr Orlando Moreno and Mr Peter Morgan for technical assistance and expert oversight of animal care.

References

1. Sykes M. Mixed chimerism and transplant tolerance. Immunity. 2001;14:417-424.
2. Sykes M, Preffer F, McAffee S, et al. Mixed lymphohematopoietic chimerism and graft-vs-lymphoma effects after non-myeloablative therapy and HLA-mismatched bone marrow transplantation. Lancet. 1999;353:1755-1759.
3. Slavin S. New strategies for bone marrow transplantation. Curr Opin Immunol. 2000;12:542-551.
4. McCarthy NJ, Bishop MR. Nonmyeloablative allogeneic stem cell transplantation: early promise and limitations. Oncologist. 2000;5:487-496.
5. Sykes M, Spitzer TR. Non-myeloblative induction of mixed hematopoietic chimerism: application to transplantation tolerance and hematologic malignancies in experimental and clinical studies. Cancer Treat Res. 2002;110:79-99.
6. Pelot MR, Pearson DA, Swenson K, et al. Lymphohematopoietic graft-vs-host reactions can be induced without graft-vs-host disease in murine mixed chimeras established with a cyclophosphamide-based non-myeloablative conditioning regimen. Biol Blood Marrow Transplant. 1999;5:133-143.
7. Mapara MY, Pelot M, Zhao G, et al. Induction of stable long-term mixed hematopoietic chimerism following nonmyeloablative conditioning with T cell-depleting antibodies, cyclophosphamide, and thymic irradiation leads to donor-specific in vitro and in vivo tolerance. Biol Blood Marrow Transplant. 2001;7:646-655.
8. Sykes M, Sheard MA, Sachs DH. Graft-versus-host-related immunosuppression is induced in mixed chimeras by alloresponses against either host or donor lymphohematopoietic cells. J Exp Med. 1988;168:2391-2396.
9. Tomita Y, Khan A, Sykes M. Mechanism by which additional monoclonal antibody injections overcome the requirement for thymic irradiation to achieve mixed chimerism in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. Transplantation. 1996;61:477-485.
10. Tomita Y, Sachs DH, Khan A, Sykes M. Additional mAb injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mAbs and 3 Gy whole body irradiation. Transplantation. 1996;61:469-477.
11. Sykes M, Sheard M, Sachs DH. Effects of T cell depletion in radiation bone marrow chimeras, I: evidence for a donor cell population which increases allogeneic chimerism but which lacks the potential to produce GVHD. J Immunol. 1988;141:2282-2288.
12. Sykes M, Abraham VS, Harty MW, Pearson DA. IL-2 reduces graft-vs-host disease and preserves a graft-vs-leukemia effect by selectively inhibiting $CD4^+$ T cell activity. J Immunol. 1993;150:197-205.
13. Unkeless JC. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J Exp Med. 1979;150:580-596.
14. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. J Immunol. 1999;162:352-358.
15. Mombaerts P, Clarke AR, Rudnick MA, et al. Mutations in T-cell antigen receptor genes a and b block thymocyte development at different stages. Nature. 1992;360:225-231.
16. Mapara MY, Kim Y-M, Wang S-P, et al. Donor lymphocyte infusions (DLI) mediate superior graft-versus-leukemia (GvL) effects in mixed compared to fully allogeneic chimeras: a critical role for host antigen-presenting cells. Blood. 2002;100:1903-1909.
17. Mapara MY, Kim Y-M, Marx J, Sykes M. DLI-mediated GVL effects in mixed chimeras established with a non-myeloablative conditioning regimen: extinction of GVL effects coincides with loss of alloreactive cells following conversion to full donor chimerism. Transplantation. 2003;76:297-305.
18. Welniak LA, Blazar BR, Anver MR, Wiltrout RH, Murphy WJ. Opposing roles of interferon-gamma on $CD4^+$ T cell-mediated graft-versus-host disease: effects of conditioning. Biol Blood Marrow Transplant. 2000;6:604-612.
19. Giralt S, Hester J, Huh Y, et al. CD8-depleted donor lymphocyte infusion as treatment for relapsed chronic myelogenous leukemia after allogeneic bone marrow transplantation. Blood. 1995;86:4337-4343.
20. Alyea EP, Soiffer RJ, Canning C, et al. Toxicity and efficacy of defined doses of $CD4^+$ donor lymphocytes for treatment of relapse after allogeneic bone marrow transplant. Blood. 1998;91:3671-3680.
21. Soiffer RJ, Alyea EP, Hochberg E, et al. Randomized trial of $CD8^+$ T-cell depletion in the prevention of graft-versus-host disease associated with donor lymphocyte infusion. Biol Blood Marrow Transplant. 2002;8:625-632.
22. Ildstad ST, Wren SM, Bluestone JA, et al. Effect of selective T cell depletion of host and/or donor bone marrow on lymphopoietic repopulation, tolerance, and graft-vs-host disease in mixed allogeneic chimeras (B10 + B10.D2 → B10). J Immunol. 1986;136:28-33.
23. Lapidot T, Lubin I, Terenzi A, et al. Enhancement of bone marrow allografts from nude mice into mismatched recipients by T cells void of graft-versus-host activity. Proc Natl Acad Sci U S A. 1990;87:4595-4599.
24. Martin PJ. Donor CD8 cells prevent allogeneic marrow graft rejection in mice: potential implications for marrow transplantation in humans. J Exp Med. 1993;178:703-712.
25. Martin PJ. Influence of alloreactive T cells on initial hematopoietic reconstitution after marrow transplantation. Exp Hematol. 1995;23:174-179.
26. Champlin R, Ho W, Gajewski J, et al. Selective depletion of $CD8^+$ T lymphocytes for prevention of graft-versus-host disease after allogeneic bone marrow transplantation. Blood. 1990;76:418-423.
27. Sprent J, Surh CD, Agus D, et al. Profound atrophy of the bone marrow reflecting major histocompatibility complex class II-restricted destruction of stem cells by $CD4^+$ cells. J Exp Med. 1994;180:307-317.